The immunomodulatory effects of probiotics.

Omakobia, Michael

Swansea University
Prifysgol Abertawe

The Immunomodulatory Effects of Probiotics

Michael Omakobia
B.Sc. M.Sc. PGCE

Submitted to the University of Wales,
College of Medicine in fulfilment of the requirements
for the degree Doctor of Philosophy

2015

THESIS ABSTRACT

Background

The development of childhood atopy is postulated to be influenced by perinatal microbial exposure. Inadequate numbers of probiotic organisms forming the intestinal microbiota, is an early pre-symptomatic feature linked to the expression of allergy. Perinatal probiotic supplementation is therefore hypothesized to contribute to the microbiota mediated immunomodulatory response determining immune hypo-responsiveness to allergens.

Aims

This thesis examined the anti-allergy immunomodulatory effects of probiotic bacteria *in vitro* in the context of the pathophysiology of atopy, as a preamble to perinatal probiotic supplementation.

Methods

Cytokine responses were measured after umbilical cord blood mononuclear cells (CBMCs) were co-cultured in the presence of a consortium of probiotic organisms consisting of two strains of lactobacilli and two strains of bifidobacteria. Additionally pregnant women with a familial history of allergic disease from 36 weeks gestation and their infants to age 6 months, were supplemented with probiotics or a placebo daily. The immunomodulatory effects of probiotics on immune function *in vivo* was then analysed within the peripheral blood of the supplemented neonates.

Results

CBMCs co-cultured in the presence of the probiotic consortium generated a dose dependent, monocyte mediated release of the pro-inflammatory cytokines TNF-α, IL-12p70, IFN-γ and the immunosuppressive cytokines IL-10 and TGF-β1. The consortium down regulated PHA and SEB induced IL-13 (a key allergy orchestrating cytokine) while potentiating IFN-γ (a key Th1 driving cytokine) responses from CBMCs. Interestingly in the probiotic supplemented group the cord blood eosinophil count was significantly reduced. Additionally the IL-12p70 concentrations in microbial stimulated venous blood at age 6 months was significantly elevated in comparison to the placebo supplemented group.

Conclusion

The immunomodulatory effect of probiotic bacteria is marked by a capacity to promote a Th1 orientation of the immune system. Probiotics administered during pregnancy and early infancy may therefore be effective in the prevention of Th2 mediated atopic disorder.

ACKNOWLEDGEMENTS

Foremost, I would like to thank GOD ALMIGHTY for enabling me to persevere to end to complete this PhD. I would also like to express my sincere gratitude to my advisor Prof Martin Sheldon for his continuous support during my Ph.D. study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance has helped me most critically during my composition and writing of this thesis.

Besides my advisors, I would like to thank the Industrial Partner Cultech for their financial support during my studies. I particularly want to thank Dr Iveta Gariova and Dr Sue Plummer for their encouragement and insightful comments, and input during my thesis write up.

My sincerest thanks also goes out to Swansea University, for offering me the opportunity to study for a Ph.D. in such a beautiful city. I am also grateful to the Swansea Harriers whose athletics facilities allowed me to pursue my passion for sprinting and allowed timely rests from my intense PhD research.

I also want to thank Mr Chris Ravenhill for his expert advice at a crucial point during my studies, without his input completion would have not been possible. I would also like to thank my closest friends Pauline Araba Addison, Hazel Mckenzie and Jide for their inspiring speeches at the most timely points of my study. Their words encouraged me not to give up on the programme.

I also want to thank Prof Morgan and Prof. Thornton for an inadvertent, yet highly motivational final meeting I had with them as data analysis had reached a natural conclusion. The words of this meeting became the driving force for successful completion of my Ph.D.

Last but not the least; I would like to thank my family for their undying support throughout my life. I would like to thank my gorgeous fiancée and our lovely daughters Salma and Azariah who have been a major inspiration. For this reason when they are older I want them to know three things regarding my PhD story. All things are possible with GOD. The P in Ph.D. actually stands for perseverance. And that diamonds require pressure to form.

List of figures:

List of Tables:

List of Abbreviations

APC: Antigen presenting cells
BSA: Bovine serum albumin
beta
CBMCs: Umbilical cord blood mononuclear cells
CD: Cluster of differentiation
CD235a: Glycophorin A
CFU: Colony forming units
CI: Confidence intervals
CM: complete medium'
CSFE: Carboxyfluorescein diacetate succinimidyl ester
ConA: Concanavalin A
DAF: Decay accelerating factor
DC: Dendritic cells
ECP: Eosinophil cationic protein
ELISA: Enzyme linked immunosorbent assay
FACs: Fluorescence activated sorting
FBS: Foetal bovine serum
FcεRI: High affinity IgE Receptor
FOS: Fructooligosaccharides
FoxP3: Foxhead box P3
GALT: Gastrointestinal associated lymphoid tissue
GF: Germ free mice
GIT: Gastrointestinal tract
GOS: Galactooligosaccharides
IDO: Indoleamine 2, 3,-dioxygenase
IEC: Intestinal epithelial cells
IgA: Immunoglobulin A
IgE: Immunoglobulin E
INF-γ: Interferon gamma
IL: Interlukin
IQR: Interquartile range
ISAAC: International Study of Asthma and Allergies
LGG: *Lactobacillus rhamnosus GG* (ATCC 53103)
LPS: Lipopolysaccharide
MAMPs: Microbial associated molecular patterns
MCP: Membrane co-factor protein
mDCs: Myeloid dendritic cells
MHC: Major Histocompatibility complex
MLN: Mesenteric lymph nodes
MNCs: Mononuclear cells
MRD: Maximum recovery diluent
MRS: MRS agar
OR: Odds ration
PBMCs: Peripheral blood mononuclear cells
PBS: Phosphate buffered saline
pDCs: Plasmacytoid dendritic cells
PGN: Peptidoglycan
PHA: Phytohaemagglutinin
PRR: Pattern recognition receptors
RBCs: Red blood cells
RDBPC: Randomised double blind placebo controlled
RPM: Rotations per minute
SCORAD: Scoring atopic dermatitis
SEB: Staphylococcal enterotoxin B
SPTs: Skin prick tests
T_0: Time 0
T_{24}: Time 24
T_{48}: Time 48
Th1: T helper 1
TGF-β: Transforming growth factor

TLR: Toll like receptor
TVCs: Total viable counts
TNF-α: Tumor necrosis factor alpha

Chapter 1: Introduction

1.1 History of the Probiotic concept

The word probiotic originates from the Greek words pro and bios meaning "for life" to describe a family of commensal bacterial that:

(1) Colonise the mammalian gastrointestinal tract (GIT).

(2) Improve the microbial balance

(3) Impart a health benefit surpassing that of general nutrition alone.

In their active state probiotic bacteria are recognised as antonyms of antibiotics due to their growth stimulatory effects on other bacterial microorganisms (Guarner and Schaafsma 1998). They are non-pathogenic, and thrive symbiotically in the presence of the mammalian cellular immune system, imparting an immunomodulatory influence on the host immune equilibrium.

The diverse array of probiotic bacteria within the GIT occurs in a niche specific manner determined by their metabolic and survival needs and their immunogenicity renders them far from immunologically inert. This allows an inadvertent contribution to the microbiota-immune axis impacting upon both immunologic and non-immunologic facets of human immunity and shaping both local and systemic immunomodulatory responses.

Although no legal definition for the term "probiotic" exists the minimum criteria for classification suggests a probiotic must be:

(1) Viable.

(2) Delivered in optimal dosages with minimal batch to batch variation through shelf life.

(3) Specified at genus and strain level

(4) Efficacious when administered in specific doses in controlled studies.

Probiotic supplementation is therefore expected to contribute to gastrointestinal steady state homeostasis:

The rationale behind perinatal probiotic supplementation of the newborn stems from observations that probiotic bacteria colonise the neonate immediately after birth [See 1.5.4]. Subsequently the immunomodulatory effects of probiotic bacteria is now a key area of interest in contemporary immunology, an interest compounded by the

premise that a probiotic optimised GIT is widely connected with the resistance of the allergic phenotype [See 1.5.1].

The earliest reported reference to probiotic bacteria occurs in a Persian version of the book of Genesis 18:8 emphasising that Abraham owed his longevity to the consumption of fermented "soured milk" (Schrezenmeir and de Vrese 2001; Crane 2002). The notions that ingestion of large inoculums of probiotic bacteria, of lactic acid bacteria (LAB) and bifidobacteria origin, improves gastrointestinal and immune health have been prevalent since the 1900s. This era witnessed the advent of dietary intervention methods employing probiotic bacteria enriched dairy products to improve health and increase longevity of human life (Gill 1998).

Bifidobacteria were first described and isolated by French paediatrician, Dr Henry Tissier in the 1900s as the predominant organism in the stools of breast-fed infants and named *B. bifidus* (Tissier 1900). Rapid colonisation of the newborns' GIT with lactic acid producing bifidobacteria and lactobacilli strains under the influence of breast milk is now a widely accepted paradigm (Crane 2002). Tissier (1990) hypothesised that bifidobacteria are crucial in preventing the outgrowth of pathogenic diarrhoea inducing bacteria by abrogating their ability to grow via a phenomenon known as competitive exclusion.

In 1908 Metchnikoff hypothesized that ingestion of large quantities of LAB (probiotic supplementation) with intrinsic fermentative capacity increased human longevity, and that consumption of foods initially fermented with probiotic bacteria conferred a desirable health benefit via regulation of the intestinal autointoxication process. Anticipated health benefits were attributable to improvements to the GIT microbiota balance due to replacement of proteolytic putrefying organisms, such as *Clostridium* (potent producers of toxic substances like ammonia from protein metabolism), with more useful fermentative lactic acid producing bacteria that exponentially outgrow the growth of pathogenic organisms and restricting the growth of toxin-producing bacteria due to direct competition for nutrients vital for survival within the GIT niche (Metchnikoff 1908).

In 1920 Rettger and colleagues highlighted that *Lactobacillus bulgaricus* had a poor survival rate upon transit through the GIT and hypothesised that organisms isolated from human stools were superior probiotics, being more amenable to GIT transit and exponential growth and colonisation during transit (Rettger *et al.* 1935). In 1930 a human faecal isolate of *Lactobacillus acidophilus* was used in a commercial milk fermented product named acidophilus milk (Rettger *et al.* 1935) and similarly in 1935 the Japanese food company, Yakult Honsha, commercialised the sale of live bacteria in a fermented milk drink. This arose from the discovery of *Lactobacillus casei Shirota* capable of transiting the acidic environment of the stomach and colonising the intestine. This organism was subsequently introduced into a fermented milk drink named Yakult which is now sold worldwide (Yalkult 2010).

1.1.1 Lactic acid bacteria (LAB) and bifidobacteria in health and immunity

Probiotic bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* represent major bacterial taxa utilised for probiotics (See Table 1.1). Lactobacilli are Gram-positive, catalase-negative bacteria capable of producing lactic acid as a primary end-product of the fermentation of carbohydrates. The genus *Bifidobacterium* is only poorly phylogenetically related to genuine LAB, and its species employ a metabolic pathway for the degradation of hexoses different from those described for LAB (Felis and Dellaglio 2007).

Bifidobacterium are Gram-positive, curved, and often bifid rods. In bottle-fed infants, however, Gram-positive, straight, unbranched rods were the predominant organisms and were identified as *B. acidophilus.* The predominance of bifidobacteria in the GIT of healthy infants during the first weeks of life was noted by Bullen (1976). Interestingly bifidobacterial numbers are fewer in the stools of bottle-fed versus breast-fed infants and once weaned these infants harbour bifidobacteria only in small numbers compared to their breast fed counterparts (Poupard *et al.* 1973). Infants fed bottle or supplementary cow's milk, commonly harboured *Escherichia coli, Streptococcus faecium, Pseudomonas aeruginosa* strains along with clostridia and proteus species. (Bullen 1976). These bacterial colonisation differences are postulated to reflect the different pH of stools from breast- versus bottle-fed infants: pH 8–9 of bottle fed infant faeces compared with the pH 5–6 of breast fed infants.

Table 1.1: Commonly employed probiotic strains of the human GIT.

Low G+C Gram-positive LAB	Bifidobacteria
L.casei	*B.infantis*
L.acidophilus	*B.bifidus*
L.crispatus	*B.angulatum*
L.brevis	*B.breve*
L.delbrueckii group	*B.longum*
L.fermentum	*B.adolescentis*
L.gasseri	*B.catenulatum*
L.paracasei	*B.pseudocatenulatus*
L.plantarum group	
L.rhamnosus	
L.ruminis	
L.sakei	
L.salivarius	
L.vaginalis	
L.curvatus	
L.fructivorans	
Pediococcus pentosaceous	
Leuconostoc argentinum	
Leuconostoc mesenteroides	

1.1.2 Toll like receptor (TLR) – mediated immunomodulatory effect of probiotic bacteria

Despite the rise to prominence of probiotic bacteria as health promoting supplements, a paucity of research data exists characterising their interactivity with the immune system and their immunomodulatory contribution to immune integrity in health and disease. This has led to heightened research interest into the immunomodulatory roles of probiotic bacteria in IgE mediated atopic disorder, an interest that forms the central theme of this thesis.

Probiotics are postulated to directly or indirectly influence immunity via uptake by the GIT M cells and subsequent transfer to antigen presenting cells. The emerging paradigm implies that probiotic bacteria can modulate the Th1/Th2 balance culminating in increased IgA producing B cells, concomitant with reduction in IgE producing cells in turn leading to a reduced allergic response (Ouwehand *et al.* 2002).

Since TLR mediated immune recognition of probiotic bacteria by innate and/or adaptive cells of immunity culminates in upregulation of immune response shaping cytokines this interaction is referred to as the immunomodulatory effect of probiotic bacteria, to define improvements in intestinal microbial balance, that impact upon host immunity both locally at the gastrointestinal associated lymphoid tissue (GALT) and systemically (Isolauri *et al.* 2001). Probiotics have therefore gained recognition as powerful inductors of the immune response (Schrezenmeir and de Vrese 2001).

Pattern recognition receptors (PPRs) e.g. TLRs expressed on the surface of APCs recognise micro-organism associated molecular patterns (MAMPs) typically bacterial cell wall components expressed on Gram-positive probiotic bacteria culminating in a downstream signalling cascade that activates transcription factors involved in cytokine signalling. The TLR system is an immune-surveillance system whose archetypal role is to recognise distinct microbial patterns of both non-pathogenic and pathogenic micro-organisms. Probiotic bacteria are recognised as non-pathogenic by

the immune system and impose a profound non-pathological immunomodulatory influence on newborn immunity that ultimately shapes the cellular architecture of the gastrointestinal associated lymphoid tissue **(GALT)** and the functionality of both the peripheral and systemic immune system.

Despite the premise that the overall number of genes in the microbiota is approximately two orders of magnitude greater than the gene number in host's DNA, microbiota-associated clinical pathology in healthy individuals does not ensue (Berg, 1996). This highlights an intrinsic evolutionary requirement for peaceful coexistence between the host and probiotic bacteria, representing a classic example of mutualism existing irrespective of the plethora of bacterial MAMPS engaged by innate and adaptive immune mechanisms (McCracken and Lorenz 2001).

Since a relationship between the qualities of probiotic bacteria colonising the neonatal microbiota and the development of atopy exists; the strain specific immunomodulatory activity of probiotic bacteria has been utilised in numerous perinatal dietary supplemental studies to treat symptoms and/or prevent disorders of an atopic derivation (See Table 1.6.1). Kalliomaki's ground breaking study in Scandinavia demonstrating that the rate of atopy was reduced by almost half in a probiotic group compared to the placebo group increased interest in the application of perinatal probiotic supplementation for the prevention of IgE-mediated allergy. This rationale is now supported by well documented studies reporting immunomodulatory benefits in both prevention (Kalliomaki *et al.* 2001) and treatment (Isolauri *et al.* 2000; Bjorksten 2004) of allergic disease. Probiotic strains of lactobacilli and bifidobacteria origin in particular have been distinguished for their anti-allergy properties (Kalliomaki and Isolauri 2004).

1.1.3 The PROBAT trial

The PROBAT trial was a randomised double blind placebo controlled study involving probiotic supplementation of expectant lactating woman from 36 weeks of gestation until their newborn was 6 months of age. Mothers of newborn recruits with a familial history of allergy from the South Wales region were preferentially selected for the trial based upon answers on the study recruitment questionnaire to assess for familial history of allergy. The trial was conducted employing a study consortium inclusive of

four probiotic strains *Lactobacillus paracasei, Lactobacillus salivarius, Bifidobacteria bifidum and Bifidobacteria lactis susp. animalis* prepared by the industrial sponsor administered daily as a probiotic supplement to both mother and newborn.

1.1.4 Aim of study and thesis hypothesis

The principal goal of this thesis was to characterise the *in vitro* and *in vivo* immunomodulatory role of a subset of probiotic bacteria emerging from the lactobacillus and bifidobacteria family in protecting against the onset/and or symptoms of IgE mediated atopic disorder). Another major goal was to contribute to our current understanding of the impact of probiotic supplementation on immune function in both *in vitro* and *in vivo* scenarios, and to characterise the immunomodulatory mechanisms employed by probiotics during development of a functionally mature immune system in the neonate. We hypothesised that perinatal probiotic supplementation would contribute to both pre and post-natal factors programming normal development of neonatal immunity during and after pregnancy marked by enhanced development of neonatal cellular immunity underlying resistance to development of childhood atopy.

1.2 Epidemiology and immunology of allergy

Current epidemiological data indicate that the prevalence of allergic diseases has risen dramatically in many parts of the developed world with approximately one in five children in industrialized countries debilitated with allergic diseases ranging from asthma, allergic rhinitis or atopic dermatitis (Jarvis and Burney 1998). This ratio is steadily increasing (Masoli *et al.* 2004) with the prevalence of diagnosed asthma and associated symptoms strongly in children increasing at a rate of about 5% a year in the United Kingdom, the Scandinavian regions, Switzerland, the United States, Australia, New Zealand, and Taipei (Jarvis and Burney 1998).

To provide further insight into the worldwide allergic epidemic, the International Study of Asthma and Allergies in Childhood **(ISAAC)**, formed in 1991 to facilitate research into the potential causes of the rise in asthma, allergic rhinitis and eczema investigated variations in the prevalence of these conditions at the population level.

Although familial associations of allergic disease indicates a genetic element as a principal causative factor, other evidence implicates a role for environmental factors. Furthermore probiotic intervention studies reveal an important immunomodulatory role for probiotic bacteria in shaping the development of the immune system and modulating pathways of immunity that impact on normal regulation of the allergic immune response (See Table 1.6).

Allergy was established as a clinically relevant ailment in 1906 by Viennese paediatrician Clemens Von Pirquet who distinguished a subset of patients who were abnormally hypersensitive to normally innocuous environmental allergens like dust, pollen or food antigens. Pirquet coined the phenomenon "allergy" to describe a rapidly developing immune reaction mounted against harmless ubiquitously occurring allergens. In the modern era allergy is regarded as a type I hypersensitivity reaction representing an instantaneous allergic response towards allergens to which individuals are atopically sensitised.

The concept of atopy, derived from the Greek meaning idiosyncratic, was applied by Coca in 1923. Atopic disorders encompass an archetypal spectrum of allergic diseases including eczema, allergic rhinitis and asthma occurring when the immune system is dysregulated. A standardised nomenclature of allergy was proposed by the European Academy of Allergy and Immunology in 2001 in which atopy is to be defined as a familial tendency to produce IgE antibodies in response to low doses of allergens, culminating in symptoms such as asthma, rhinoconjunctivitis, or eczema/dermatitis (Johansson and Lundahl 2001; Johansson *et al.* 2004). Therefore atopy is reserved for describing clinical traits and a genetic predisposition to become IgE-sensitised to environmental allergens, and is applied exclusively if IgE-sensitisation has been documented by a positive skin prick test or by IgE antibodies in serum. In contemporary immunology atopy is classified as an inherited hypersensitivity immune reaction of T helper 2 (Th2) exuberance (See T cell subsets in allergy 1.2.1) orchestrated by a limited number of allergens, mediated by excessive production of IgE antibodies towards environmental allergens (Peisong 2001). The understanding of T cell responses in allergy highlighted the critical importance of Th2 cells in helping B cells toward IgE expression. Th2 cells interact with eosinophils via IL-5; smooth muscle cells via IL-9; and epithelial cells and keratinocytes via IL-13 (Dillon *et al.* 2005; Chattopadhyay *et al.* 2007).

1.2.1 T cell subsets in allergy

Originally two T helper (Th) T cell subsets originating from the naive T cell compartment were first identified based on their cytokine secretion profile. These were termed the Th1 and Th2 T cells. The Th2- IL-3, IL-4, IL-5, IL-9 and IL-13 secreting T cells are responsible for orchestrating expression of humoral/antibody responses such as IgG or IL-4 mediated IgE induction. (Schmidt-Weber *et al.* 2007). While the Th1: IFN-γ secreting cells are crucial in inflammatory delayed type hypersensitivity responses, macrophage activation and induction of MHC class II molecules.

The Th2 cytokines IL-4, 5, 9 and 13 are important during initiation, maintenance and amplification of allergic inflammation in allergy afflicted organs (Romagnani 2000; Spergel and Paller 2003; Romagnani 2004). The discovery of further T cell subsets such as the regulatory T cells (Tregs) and Th17 has modified perceptions of atopic immunopathology and the bivalent concept of the Th1/Th2 paradigm. (Schmidt-Weber *et al.* 2007). Key observations leading to this reappraisal include the bi-phasic nature of the Th1/Th2 contribution during the pathogenesis of eczematous lesions (Grewe *et al.* 1998; Miraglia del Giudice *et al.* 2006; Ong and Leung 2006), and the inverse association between IgE mediated helminth infection and Th2 mediated allergic disease (Smits *et al.* 2010).

Th17 cells produce pro-inflammatory cytokines such as IL-17, IL-6, TNF-α, IL-1β and IL-22 (Harrington *et al.* 2005; Park *et al.* 2005; Chung *et al.* 2006; Mangan *et al.* 2006) and induce expression of inflammatory mediators, like granulocyte colony stimulating factor, acute phase proteins, and TNF-α (McAllister *et al.* 2005; Komiyama *et al.* 2006; Harrington *et al.* 2005). Thus, IL-17 production provides a means by which the adaptive immune system communicates with the innate immune system to promote inflammation (Harrington *et al.* 2005, 2006). The role of Th17 cells in allergy still requires clarification, with experimental models implicating a role for Th17 cells in mediating neutrophilic inflammation in acute airway inflammation and acute asthma attacks (Hoshino *et al.* 2000; Hellings *et al.* 2003; Nakae *et al.* 2003; Prause *et al.* 2004; Hashimoto *et al.* 2005). Interestingly, IL-17 expression is increased in the lungs, sputum, bronchoalveolar lavage fluids, or sera of

asthmatic patients, and the severity of airway hypersensitivity in patients has been demonstrated to correlate with IL-17 expression level (Molet *et al.* 2001; Chakir *et al.* 2003). IL-17 mediated neutrophilia has also been observed in bronchoalveolar lavage fluids. Interestingly sputum IL-17 mRNA was demonstrated to correlate with CXCL8 and neutrophil counts (Bullens *et al.* 2006). Furthermore anti-IL-17 has been demonstrated to reduce neutrophil infiltration in models of experimental asthma while increasing eosinophil infiltration (Hellings *et al.* 2003; Sergejeva *et al.* 2005). Conversely, exogenously administered IL-17 reduced pulmonary eosinophil recruitment and bronchial hyperactivity. Taken together, these studies suggest a regulatory role for IL-17 during allergic inflammation (Schnyder-Candrian *et al.* 2006). However, few studies assessing the impact of perinatal probiotic supplementation on maturation of the Th1, Th2 and Th17 arms of newborn immunity exist during this critical preventative window for interventions against allergic disease (Kalliomaki *et al.* 2001; Kalliomaki *et al.* 2003).

1.3 Immunology of atopy with special reference to the immunomodulatory role of probiotic bacteria in shaping current and emerging T helper paradigms

Neonatal differentiation of haematopoietic cells into lymphocyte subsets of the T cell lineage is in part controlled by alterations affecting the epigenetic status of immature thymocytes during maturation. This process is influenced by microbiota-induced cytokine responses that shape the cytokine immune microenvironment during immune homeostasis. These mechanisms coalesce to determine the type and or intensity of Th1, Th2, Th17 and T regulatory immune pathways engaged by the maturing fetus in response to microbial stimuli. For instance, newborns are prone to intracellular infections since the IFN-α promoter in the neonate is hyper-methylated to circumvent toxic and deleterious Th1 immune response. Immune mechanisms pertaining to efficient resolution of intracellular infection develop much later in infant life. Therefore specific cytokine responses are developmental stage dependent and will determine how newborns respond to perinatal probiotic supplementation with age.

At a time when the newborn is solely reliant on maternal Ig antibodies and regulatory cytokines present in human breast milk to maintain immunity (Chirico 2005); probiotic bacteria provide a strong immunomodulatory influence (Strachan 2000;

Prescott 2003) during activation of immune pathways shaping post-natal immunity. A probiotic optimised newborn intestinal tract is postulated to play an important role in attenuating the risk of Th2-mediated atopic disorder (Martinez 1994; Holt *et al.* 1999)

1.3.1 Immunology of atopy

To reverse the allergic potential of an individual two avenues have been undertaken; one method attempts to prevent the immune response from becoming Th2 skewed via perinatal intervention studies employing the use of probiotics, prebiotics or synbiotics Furrie (2005). The other method attempts to manage the individual who is already atopic (Helin *et al.* 2002; Rosenfeldt *et al.* 2003; Wang *et al.* 2004). To determine the potential efficacy of any such treatments, knowledge of the target cells and molecules of the immune response which need to be under the immunomodulatory influence of probiotic bacteria is required (Furrie 2005).

The rationale behind probiotic supplementation of the newborn stems from a hypothesis that atopic disorder is the result of a fundamental failure of immune regulation involving inadequate induction and/or activation of regulatory signalling pathways occurring via the TLR system of innate immunity. This failure is postulated to be due to a lack of early life non-pathogenic microbial stimulation from intestinal probiotic bacteria engaging TLR-mediated immune responses (See 1.1.2). This phenomenon is coined under the umbrella of the Hygiene Hypothesis introduced by Strachan (1989).

Atopic sensitisation is a fundamental feature underlying allergic dysregulation defined by positive skin prick tests involving specific IgE directed against common allergens (Kurukulaaratchy *et al.* 2005). The highest risk of childhood atopy has been associated with familial history and early life sensitisation to allergen in early life (Illi *et al.* 2001b). Development of sensitisation requires allergen exposure and repeated allergen exposure is likely to trigger symptoms (Sporik *et al.* 1990). The presence of allergen-specific IgE underlies the allergic reaction and clinical symptoms of disease. Secreted IgE circulates in the blood and binds FcεRI expressed on the surface of mast cells and basophils. Later re-exposure to the same allergen results in the allergen binding to IgE molecules on the surface of the mast cells or basophils inducing cross-

linking FcεRI and activation of the cells. Activated mast cells and basophils undergo a process called degranulation culminating in the release of pharmacologically active inflammatory mediators for e.g. cytokines, from their granules into the surrounding tissue mediators that cause the classic systemic symptoms of atopy such as vasodilatation, mucous secretion, and smooth muscle contraction and itchiness (Gold and Kemp 2005). Atopic sensitization commonly occurs early in life, if epithelial barrier integrity is compromised, and the epithelium becomes aberrantly activated. This may occur through a complex interplay of environmental insults and host factors (Cookson 2004). Barrier dysfunction in the lung and skin allows allergens to activate the epithelium and produce cytokines that are permissive for the induction and development of Th2 responses (Fort *et al.* 2001; Prefontaine *et al.* 2009; Paul and Zhu 2010). A dysfunctional epithelial barrier also allows antigen-sampling dendritic cells to become directly activated by allergens and undergo maturation in a Th2-permissive milieu such that they subsequently prime allergen-specific Th2 responses (Cookson 2004; Paul and Zhu 2010; Islam and Luster 2012). Key hematopoietic effector cells in allergy include T lymphocytes, B lymphocytes, dendritic cells, and eosinophils.

Signature immunological features of atopic disorder include elevated production of the Th2 cytokines IL-4, 5, 9 and 13 accompanied by a decrease or similar production of the Th1 cytokine IFN-γ (Bottcher *et al.* 2006). While attenuated IFN-γ production at birth is considered a hallmark of the immune response of those who develop atopy, older allergically sensitised children present with hyper-production of IFN-γ while others may have mixed Th1/Th2 responses variations of which can be related to different patterns of allergic disease (Macaubas *et al.* 1999; Smart and Kemp 2002; Heaton *et al.* 2005). Therefore, a detailed understanding of the kinetics of T cell immunity underlying atopic sensitisation is necessary before one can decide how best to employ perinatal probiotic supplemental strategies to manipulate the immune response in clinically predisposed atopic individuals (Prescott *et al.* 1999).

1.3.2 Role of allergens

Allergens are diverse antigens capable of triggering allergic reactions and IgE sensitisation within breached tissues. They can be presented to the immune system in minute doses across the epitheliums of the respiratory and gastrointestinal tracts and

also the skin. Some allergens (e.g. house dust mite proteins) exhibit protease activity demonstrated to be inhibited by probiotic bacteria (Ichikawa *et al.* 2009).

1.3.3 Immunoglobulin E and the allergic reaction

The discovery of the immunoglobulin E (IgE) antibody class served as a major breakthrough in the understanding of the pathogenesis of allergy (Ishizaka *et al.* 1966). Granulocytes, particularly the mast cells initiate the allergic response via signal transduction through their high-affinity (IgE) receptors (FcεR1) together with membrane-bound surface IgE when directly engaged with allergens of the external environment. Signals initiated by IgE-mediated allergenic interactions are transduced via the intrinsic expression of FcεR1 on the granulocytes cell surface (Oettgen and Geha, 2012).

The evolutionary role of IgE in humans is to provide protective immunity against parasitic worms once endemic in the economically developed world; however in the absence of a parasitic burden in affluent societies, IgE mediated immunity to allergens has seemingly taken precedence (Bell 1996). Early immunological mechanisms predisposing an individual towards IgE mediated atopy are complex and instrumental to development of allergic disease.

1.3.4 Role of the granulocytes in allergy and innate immunity

Th2 cytokines are instrumental to the allergic response which concludes in recruitment of granulocytic effector cells **(See Figure 1.1)** such as the eosinophils, basophils and mast cells which migrate to sites of allergic tissue inflammation either alone or in combination with cytophilic or IgE antibodies promoting the clinical manifestations of atopy (Cross *et al.* 2001).

Upon recruitment from the circulation the granulocytes localise to sites susceptible to allergic challenge such as the epithelial surfaces of the intestinal and respiratory tracts and the skin (Furrie 2005). This process requires upregulation of adhesion molecules on the endothelium of blood vessels and granulocytes, and the subsequent granulocyte migration through the vessel wall towards the inflammatory site along a chemokine and/or cytokine concentration gradient (Lampinen *et al.* 2004).

Human granulocytes respond to a variety of adaptive- and innate- immune stimuli that regulates de novo generation of inflammatory cytokines pivotal in allergic disease, they do not just contribute to symptoms of allergic disease but also orchestrate events leading to them (Gibbs 2005). For example, despite the basophilic granulocytes representing approximately 1% of the total nucleated peripheral blood pool (Macfarlane *et al.* 2000), basophils are not only recruited to sites of allergic inflammation but their mediators are released in sufficient quantities to contribute to allergic symptoms (Gibbs 2005). Their cellular expression of CD40 ligand synergises with IL-4 and IL-13 cytokines to induce B-cell proliferation and class switching to IgE and IgG4 (Macfarlane *et al.* 2000).

The combined application of granulocytic-specific monoclonal antibodies together with techniques to purify granulocytes to homogeneity from peripheral blood, led to the observation that key granulocyte populations interactive with T and B cells are a common occurrence in allergy afflicted tissues (Falcone *et al.* 2000; Voehringer *et al.* 2004; Gibbs 2005). For example examination of lesional skin from atopic dermatitis subjects revealed increased eosinophils counts (Oyoshi *et al.* 2009). Whereas basophils form a major infiltrate in bronchial biopsies and airway tissues of asthma patients (Koshino *et al.* 1996; Gauvreau *et al.* 2000; Macfarlane *et al.* 2000; Nouri-Aria *et al.* 2001). Furthermore basophils are the major infiltrate in nasal lavage fluids after allergen provocation from patients with allergic rhinitis (Iliopoulos *et al.* 1992) and also occur in skin biopsies from patients with atopic dermatitis (Macfarlane *et al.* 2000). This has shed light on the immune role of the granulocytes during propagation of the allergic response and immunity to pathogens.

Interestingly, granulocyte cytokine release without the need for prior IgE-mediated allergen sensitisation can occur pointing to granulocytic involvement in directing Th2 immune responses from initial pathogen or TLR agonist interaction. Bacterial peptidoglycans can similarly activate basophils by stimulating TLR2 receptors culminating in IL-4 and IL-13 release, further potentiating an IgE mediated allergic response (Oettgen and Geha 1999; Turner and Kinet 1999; Gould *et al.* 2003; Bieneman *et al.* 2005; Galli *et al.* 2005). For example, TLR2 expressing basophils upon interaction with TLR2 ligands such as (e.g., Peptidoglycan) can directly activate basophils for mediator/cytokine secretion as well as augment FcεRI-

dependent responses thus highlighting the potential of a TLR2 ligand such as peptidogoglycan expressed on probiotic bacteria to influence the innate immune response (Grangette *et al.* 2012).

With these observations in mind one major goal of this thesis was to determine whether perinatal probiotic supplementation could have any effect on circulating peripheral blood granulocyte levels. The immune role of the granulocytes during the initiation and maintenance of the allergic response is summarised in the diagram below

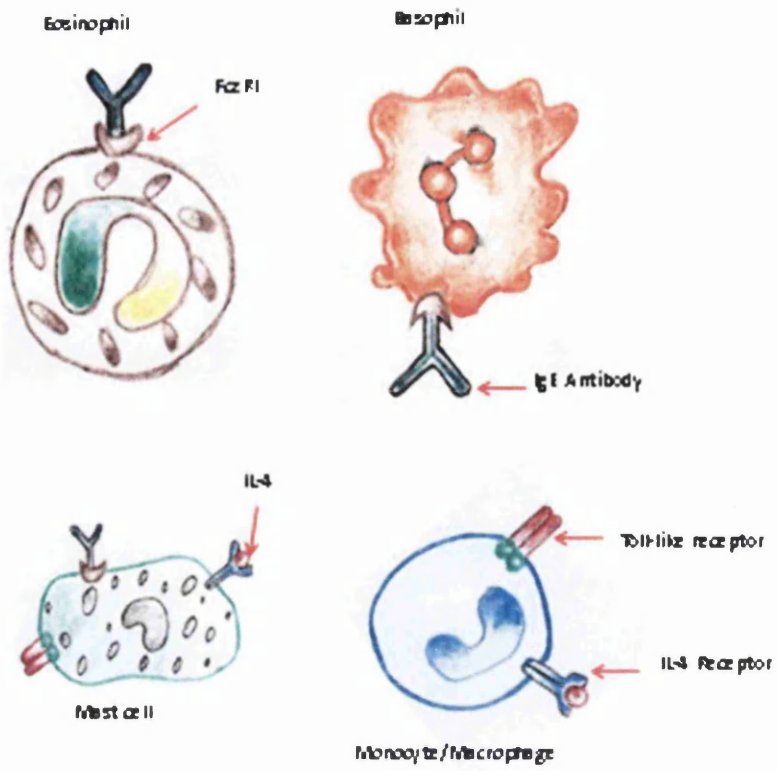


Figure 1.1 Schematic overview of the key hematopoietic effector granulocytes involved in allergy.

1.3.5 Role of the lymphocyte subsets of adaptive immunity in allergy

Lymphocyte populations form a vital cellular constituent of the adaptive immune system and disease states tend to be marked by clearly defined lymphocyte populations. Lymphocyte compositional analysis therefore serves as a useful diagnostic tool in understanding the pathogenesis of allergic disease. According to the expression levels of cell type specific, cluster of differentiation (CD) proteins, lymphocyte populations are divided into three main subgroups: The ($CD3^+$) T cells, the ($CD19^+$) B cells and the ($CD3^-/CD16^+$ $CD56^+$), natural killer cells.

Mature T cells in peripheral blood are further sub-divided into ($CD4^+$) helper T cells that release cytokines and growth factors regulating the differentiation of other immune cells and ($CD8^+$) cytotoxic T cells that exhibit suppressor activity that lyse tumorous or virally infected cells (Supramac 2008). Optimal functioning of the immune system can be influenced by imbalances resulting from abnormalities in the

number of lymphocytes in either the CD4 or CD8 lymphocyte compartments. The CD4/CD8 ratio is therefore used as a parameter to assess immune system health (Calder 2007).

The expression of alternatively spliced mRNA isoforms of CD45 a trans-membrane protein tyrosine phosphatase expressed on haematopoietic nucleated cells (Ten Dam *et al.* 2000) renders $CD4^+$ and $CD8^+$ T cells either naïve ($CD45RA^+$) or memory ($CD45RO^+$) T cells (Akbar *et al.* 1988; Sanders *et al.* 1988; Hermiston *et al.* 2003). This definition is based on the ability of $CD45RO^+$ T cells, to respond exclusively to recall antigen *in vitro*, but not $CD45RA^+$ (Akbar *et al.* 1988; Sanders *et al.* 1988; Plebanski *et al.* 1992), and the distinction that the majority of $CD4^+$ T cells in cord blood express the CD45RA antigen (Depaoli *et al.* 1988).

Naive T-cell generation by the thymus and proliferation and differentiation within the periphery provide the developing newborn with a naive T cell repertoire to mount neonatal adaptive immune response (van Gent *et al.* 2009). Although the influence of perinatal probiotic supplementation on naïve and memory T cell pools has not been typically studied, a consequence of naive T cell priming at epithelial sites is the generation of memory T cells persisting throughout the lifespan of the host after resolution of inflammation (Sheridan and Lefrancois 2011). In the context of atopic diseases, sensitization to environmental allergens at the epithelial surfaces generates long-lived allergen-specific memory T cells programmed to home to the mucosal and epithelial surfaces for immune surveillance. Re-exposure to antigen or allergen can also increase the size of the tissue resident memory T cell pool (Vezys *et al.* 2009). These programmed homing pathways increase the likelihood that tissue-patrolling memory T cells will encounter activated dendritic cells presenting a cognate allergen in the tissue as well as in the draining lymph nodes. After T cell receptor activation, naive T cells differentiate into effector T cells, some of which are maintained as memory T cells expressing an altered profile of trafficking receptors; after inflammation is resolved. Low levels of the ligands for these receptors are constitutively expressed in the steady state to facilitate tissue-specific memory T cell homing and immune surveillance (Schaerli *et al.* 2004).

Tissue-specific trafficking within the circulatory system via adhesion molecules involving lymphoid organs and peripheral tissues is a fundamental prerequisite for effector T cell function and lymphocyte extravasation into target peripheral tissues

(Luster *et al.* 2005). During inflammation, ligands for selectins, integrins and chemoattractant receptors are upregulated in tissues and provide directional cues for inflammatory T cells (on which the corresponding receptors are upregulated) for entry into inflamed tissue via the circulatory system. Allergy-prone organs such as the skin and intestinal tract provide an additional level of specificity in organ-specific T cell trafficking via a process known as 'imprinting' (Luster *et al.* 2005; Agace 2006; Bromley *et al.* 2008) best characterized in the skin and small intestine (Cahill *et al.* 1977; Picker *et al.* 1990; Berlin *et al.* 1995). Since micro-organisms are tissue tropic in virulence during initial priming of naïve T cells, allergy-prone organs exposed to the environment imprint, preferential tissue-specific inflammatory effector T cell and memory T cell entry from the circulation to streamline immune surveillance (Cahill *et al.* 1977; Agace 2006; Sigmundsdottir and Butcher 2008). T cells programmed to home to the small intestine are programmed during initial antigen priming and typically express integrin $\alpha4\beta7^{+}$.

Regulatory T cells (Tregs) maintain peripheral T-cell self-tolerance to innocuous self and or environmental proteins and exert immunological control over excessive inflammatory responses (Sakaguchi 2004). Tregs mediate immunomodulatory or immunosuppressive capacity to inhibit inappropriate and potentially deleterious immune responses.

Impairment of pathways affecting Treg populations and function are cited as central in driving the immunology of clinical atopic disease (Campbell *et al.* 2001). Pivotally, Treg dependent production of the immunomodulatory cytokines IL-10 and TGF-β plays a key role in controlling allergic inflammatory responses (Joetham *et al.* 2007; Sohn *et al.* 2007; Provoost *et al.* 2009). This is exemplified by studies demonstrating that allergen-specific immunotherapy with house dust mite antigen decreased symptoms in asthmatic children in an IL-10 dependent manner, culminating in the expansion of Treg cells (Seddiki *et al.* 2006).

In this respect, one goal of perinatal probiotic supplementation is to enhance the neonatal Treg repertoire postulated to be protective against an allergic outcome. Substantiating these observations studies in germ free (GF) mice highlighted a causal link between the intestinal microbiota and the development of gut derived Treg cells

(McLoughlin and Mills 2011). Interestingly GF mice express lower numbers of $CD4^+CD25^+$FoxP3 cells with attenuated IL-10 production kinetics in the mesenteric and peripheral lymph nodes compared with conventionally reared mice (Ostman *et al.* 2006). Furthermore, oral tolerance could not be established in GF mice because of the impaired suppressive function of their $CD4^+CD25^+$FoxP3 cells and attenuated production of TGF-β and IL-10 (Ishikawa *et al.* 2008). *In vitro* experimental evidence also exists for the direct effects of *Bifidobacterium infantis* and *Lactobacillus salivarius* (employed as constituents of the PROBAT study consortium) as inducers of increased Treg cell development (de Roock *et al.* 2010). Elevated $CD4^+CD25^+$FoxP3 Treg cells in the mice spleen were associated with *Bifidobacterium infantis* supplementation in mice (Ishikawa *et al.* 2008). Furthermore a recent phase II trial demonstrated that daily administration of *Lactobacillus salivarius* for 4 weeks in healthy adults enhanced systemic concentrations of Treg produced IL-10 (Sierra *et al.* 2010). Another study highlighted the potent effects of the intestinal microbiota on Treg cell development in human subjects and a positive correlation was found between the numbers of Gram-positive anaerobes (lactobacilli and bifidobacteria) in maternal stool and IL-10 secretion by naive cord blood mononuclear cells. Notably gram-negative anaerobes (Bacteroides and Prevotella species) were also involved in this positive correlation (Karlsson *et al.* 2002) highlighting that the immunomodulatory probiotic effect is not exclusively restricted to Gram positive anaerobes.

The role of dendritic cells (DCs) is considered particularly in this thesis due to their dendrite mediated capacity to directly interact with probiotic bacteria and mediate tolerogenic immunomodulatory cytokines responses. (DCs) originate from the bone marrow and occur either as conventional plasmacytoid DCs (pDC) or myeloid DCs (mDC). DCs are potent antigen presenting cells characterised by a high capacity for antigen uptake. They are widely distributed in the gastrointestinal tract, lymph nodes and occur in low frequencies in peripheral blood to regulate naïve T cell maturation into effector T cell populations and subsequent polarization of the T cell response. Their intrinsic cell surface expression of pathogen recognition receptors (PRRs) such as TLRs renders them (1) responsive to probiotic bacteria specific MAMPs, and (2) able to present non-probiotic bacteria derived antigens to naïve T cells upon (PRR)-mediated (Kalinski *et al.* 1999).

DCs are central to the initiation of immunological reactivity to allergens and their contiguous dendritic networks are present in the epithelium of the gut, skin and airways where they mediate antigen surveillance. Interestingly cross-sectional studies in older children and adults with atopic disorder have described changes in DC subsets in patients with asthma (Uchida *et al.* 2001; Matsuda *et al.* 2002; Hagendorens *et al.* 2003). Animal models highlight the importance of mDCs during pathogenesis of allergic airway inflammation,(Constant *et al.* 2002; van Rijt *et al.* 2005), and a role for pDCs in immune tolerance (Colonna *et al.* 2004), and host defence against viral infections at mucosal surfaces (Lund *et al.* 2006).

Gut DCs are constantly exposed to intestinal probiotic bacteria and bridge the gap between innate and adaptive gastrointestinal immunity by continually sampling gut luminal content. When immature state DCs capture antigen in the presence of appropriate inflammatory stimuli, they migrate to secondary lymphoid organs and undergo maturation via upregulation of major histocompatibility molecules required for (1) antigenic peptide presentation, (2) upregulation of co-stimulatory molecules required for T-cell stimulation (3) de novo cytokine induction necessary for orchestrating stimulus-specific enhancement of T, B and NK cell responses. At the lymph nodes DCs mediate differentiation of naive T cell into effector T cell populations critically involved in driving gut Treg differentiation and tolerogenic B- and T-cell responses (Borchers *et al.* 2009).

At the cytokine level IL-12p70 production by dendritic cells is pivotal during induction of probiotic mediated Th1 response. Whereas DCs that produce IL-10 or TGF-β promote induction of Treg cells and tolerogenic responses (Tang 2009). At the immunoglobulin level, DCs facilitate IgA secretion by B cells to maintain intestinal epithelial barrier function to commensal organisms (Macpherson *et al.* 2005).

Interestingly, DCs found in the intestinal mucosa preferentially induce differentiation of resident T cells into Th2 (Iwasaki and Kelsall 1999) and Treg (Kelsall and Leon 2005) subsets promoting a tolerogenic state in the GIT. This observation was supported by *in vitro* findings that DCs can be conditioned towards a tolerogenic phenotype by intestinal epithelial cells stimulated with *Lactobacillus spp.* gut

microbiota isolates. Interestingly Gram-negative *E. coli* were less effective in mediating this tolerogenic conditioning property (Zeuthen *et al.* 2008).

DCs from GF mice are reduced in numbers and are impaired in their ability to activate T cells (Walton *et al.* 2006). The ability of the microbiota to influence antigen presenting cell function in the gut is supported by studies indicating that bone marrow derived DCs incubated with *Lactobacillus* species had enhanced ability to promote Treg cell differentiation *in vitro* and prevent intestinal inflammation after adoptive transfer *in vivo* (Foligne *et al.* 2007). Furthermore lactobacilli *spp.* have been demonstrated to differentially regulate DCs, with consequent influence on the Th1/Th2

cytokine balance at the intestinal mucosa (Christensen *et al.* 2002) as well as on the activation of NK cells (Fink *et al.* 2007). Interestingly, exposure to non-viable heat-killed probiotic *Lactobacillus* species induced the upregulation of DC maturation markers, such as MHC class II and CD86, in murine DCs confirming that probiotic bacteria do not need to be viable to be immunogenic (Christensen *et al.* 2002).

Gut homing Lymphocytes involved in the intestinal immune response are localised to organized immune inductive sites of the GALT such as Peyer's patches (PP), mesenteric lymph nodes (MLN) and diffuse effector sites of the gut epithelium and lamina propria (LP). B7 integrins are responsible for trafficking and retention of these lymphocytes at these sites. α4 integrin (CD49d) subunit combines with the β7 integrin to form the α4 β7 integrin responsible for homing of naïve and gut tropic α4 $\beta7^+$ memory effector lymphocytes via extravasation from blood to gut mucosal tissues of GALT, MLN and LP (Pabst 1991; Briskin *et al.* 1997; Williams and Butcher 1997). These inductive sites are responsible for priming of naive B and T lymphocytes that later seed to mucosal effector sites like the gut lamina propria (Farstad *et al.* 1996; Williams and Butcher 1997; Gorfu *et al.* 2009). Thus, lymphocyte populations are retained in steady state in the lamina propria or in the epithelium of various tissues. The ability of lymphocytes to be mobile and survey on the one hand, and to localize and accumulate in tissues on the other hand are critical for host defence. T cells in nearly all mucosal epithelial sites express a4 β7 integrin demonstrated to mediate lymphocyte binding to epithelial cell E-cadherin. These

interactions are key in directing lymphocyte traffic and retention in mucosal organs (McCracken and Lorenz 2001).

B cells whose principal effector molecules are the IgE antibodies, have a pivotal role to play during development of allergic disease. Variable diverse and joining segments for the immature IgE immunoglobulin transcripts (VDJCε) are detectable in the umbilical cord blood from the third trimester becoming more prevalent after 9 months of age (Miller *et al.* 1973; Lima *et al.* 2000; Thornton *et al.* 2003) Sterile Iε transcripts can be detected as early as 8 weeks of gestation indicating that many foetuses are primed for isotype switching to IgE production by the end of the first trimester of pregnancy. Post-natal maturation of circulating and tissue B cells in relation to allergic disease outcomes is not extensively studied, but it will be interesting to note whether probiotics influence on development of post-natal B cell populations. This is considered in chapter 5 of the thesis.

B cell development occurs in the bone marrow where $sIgM^{+}SIgD^{+}$ B cells exit as immature transitional B cells. Circulating B cells at birth have an immature phenotype with the majority of cells expressing IgM and CD1c (Sims *et al.* 2005; Cuss *et al.* 2006). Immediately after birth there is an increase in recent bone marrow emigrant cells which differentiate into naive B cells. Consequently, B cells are present in ample amounts in cord blood. Naive B cells constitute the largest peripheral B cell subset throughout childhood and peak during the first 6-12 month of life decreasing gradually during early childhood (van Gent *et al.* 2009; Comans-Bitter *et al.* 1997; de Vries *et al.* 1998).
Naive B cells can differentiate into plasma or memory B cells following binding of antigen by specific immunoglobulin receptors (Pascual *et al.* 1994; Liu *et al.* 1996)

1.3.6 Key cytokines regulating allergic responses

TNF-α and **IL-12p70** are inflammatory cytokine that provide differentiative signals for polarization of naive T cell inflammatory responses towards a Th1 immune pathway. The Th1 response is antagonistic to the Th2 response (Miettinen 1996; Hehlgans and Pfeffer 2005; Uings *et al.* 2005; Kekkonen *et al.* 2008). Interestingly during allergic responses TNF-α is an important inflammatory inductor of eosinophil activation and survival (Levi-Schaffer *et al.* 1998). *In vitro* studies have

demonstrated that several Lactobacillus strains interact with mononuclear cell cultures via engagement of the NF-κB pathway, thus elevating TNF-α concentrations in cell culture supernatants (Matsuguchi *et al.* 2003; Viljanen *et al.* 2005). IL-12p70 further potentiates the Th1 immune response by stimulating NK and T cell IFN-γ production and promoting peripheral blood lymphocyte proliferation and differentiation into Th1 lymphocytes (Trinchieri 1995; Szabo *et al.* 2003; Kekkonen *et al.* 2008).

IFN-γ produced predominantly by effector lymphocyte subsets is also a Th1 cytokine involved in nearly all phases of the inflammatory response, including activation, growth and differentiation of macrophages, T, B and NK cells and is crucial for resolution of allergic-related immunopathologies (e.g. severe asthma). Suppressive effects of IFN-γ on allergic diseases are postulated to be mediated by mechanisms involving: (1) Regulation of allergen presentation to T lymphocytes. (2) Differentiation of naive T cells toward Th1 phenotype and/or inhibition of Th2 cell recruitment/differentiation. (3) Suppression of Th2 cytokine release from activated T cells. (4) Inhibition of effector cell recruitment to the site of inflammation. (5) Induction of apoptosis in T cells and eosinophils and (6) and blockage of IgE isotype switch in B cells (Teixeira *et al.* 2005). Interestingly, (Prescott *et al.* 2005) demonstrated that administration of probiotics was associated with significant increases in IFN-γ responses to PHA and SEB. The increase in IFN-γ responses to SEB was directly proportional to the decrease in the severity of atopic dermatitis.

IL-1β and **IL-6** exert both pro- and anti-inflammatory activity on monocytes, B cells and T cell , and acute phase reactions (Fitzgerald *et al.* 2001). IL-1β induces neutrophilia *in vivo* and drives the early phase of the inflammatory response and resultant changes in airway smooth muscle responsiveness in asthma patients (Whelan *et al.* 2004). Elevated levels of IL-1β protein have been detected in the airways of patients with asthma (Pujol *et al.* 1990; Borish *et al.* 1992) and IL-1β modulates airway constrictor and smooth muscle relaxation responses (Hakonarson *et al.* 1997; Shore and Moore 2002). However, the role of probiotic bacteria as an inductor of the IL-1β response has not been studied.

IL-6 induces expansion of Th2 effector cells involved in allergic responses; when released by dendritic cells and inhibits the suppressive function of $CD4^+CD25^+$ Tregs thus inhibiting peripheral tolerance (Teta 2006). In the context of the progression of allergy, IL-6 binds the soluble IL-6 receptor (IL-6R), increased after allergen challenge in asthmatic patients. Conversely via membrane-bound IL-6R, IL-6 controls $CD4^+CD25^+$ survival, and initial stages of Th2 cell development in the lung. Currently, there is a paucity of studies modelling interactions with probiotic bacteria and IL-6R but it is postulated that IL-6 may exert anti-inflammatory and protective effects in intestinal mucosa and enterocytes. (Viljanen *et al.* 2005) studied *in vivo* the immunologic effects of *Lactobacillus rhamnosus (*LGG), probiotic supplementation in infants with atopic eczema-dermatitis syndrome and cow's milk allergy for 4 weeks and discovered that plasma IL-6 levels increased significantly after treatment with LGG.

1.3.7 Role of the classical Th2 cytokines

IL-4 is a mast and T cell derived Th2 cytokine that plays a critical role in IgE regulation and stimulation of activated B and T-cell proliferation differentiation of naive helper T cells into Th2 cells (Sokol *et al.* 2008). IL-4 exerts its biological roles through the IL-4 receptor expressed on B cells, T cells, and monocytes. The IL-4-dependent production of IgE due to IL-4 induced isotype class switching of IgM to IgE (Isidoro-Garcia *et al.* 2005). Overproduction of IL-4 is associated with allergies (Sokol *et al.* 2008) and differentiation to the Th2 allergic phenotype which plays a crucial role during induction and maintenance of allergy. Asthma and atopy have been linked to the IL-4 gene mapped to chromosome 5q31 (Marsh *et al.* 1994; Palmer *et al.* 1998) and IL-4 polymorphisms are associated with increased total IgE levels and allergic phenotypes (Basehore *et al.* 2004). The IL-4RA gene is located on chromosome 16p (16p12.1) (Pritchard *et al.* 1991), a region implicated in linkage with atopy in diverse populations (Deichmann *et al.* 1998; Ober *et al.* 2000). Several single-nucleotide polymorphisms have also been identified in the coding region of the IL-4RA gene, many of them resulting in amino acid substitutions (Deichmann *et al.* 1997; Deichmann *et al.* 1998). Polymorphism of IL-4RA gene plays central role of the IL-4/IL-4RA pathway in atopy and association of the 576Q>R polymorphism with the atopic phenotype has been described (Hershey *et al.* 1997; Kruse *et al.*

1999; Sandford *et al.* 2000; Shirakawa *et al.* 2000; Kauppi *et al.* 2001; Bottini *et al.* 2002; Wjst *et al.* 2002; Hytonen *et al.* 2004; Lee *et al.* 2004).

IL-13 is an immunoregulatory Th2 cytokine produced by activated T cells that regulates immunological processes that underpin the pathophysiology of allergic disorders. IL-13 enhances expression of CD23 on monocytes and B-cells and also promotes IgE mediated allergic inflammation while inhibiting microbial driven inflammation. IL-13, like IL-4, contributes to isotype switching in B cells to promote IgE production and modulates inflammation by attenuating expression of IL-1 and TNF-α (Foster 2003). Investigations on over-expression of IL-13 in the lung and in acute models of asthma in mice have defined important roles for IL-13 in eosinophil accumulation and the promotion of enhanced airways hyperreactivity to cholinergic stimuli (Kumar *et al.* 2003; Yang *et al.* 2003; Leigh *et al.* 2004).

1.3.8 Role of the regulatory cytokines

TGF-ß1 is a potent immunoregulatory cytokine instrumental in tissue remodelling, wound repair, haematopoiesis (Fitzgerald *et al.* 2001; Marie 2006), prevention of intestinal inflammation, upregulation of IgA synthesis (Yang *et al.* 1999) and inhibition of inflammatory T and B cells, dendritic cells, mast cells and eosinophils (Wahl 1992; Holgate 2000; Solway and Irvin 2007). The onset of allergy has been attributed to a lack or decreased number of suppressive, Tregs. TGF-β has been demonstrated to induce Tregs and participate directly in suppression of effector T cells thereby facilitating the return of reactivity to allergens to normal sub symptomatic activity (Schmidt-Weber and Blaser 2004). Interestingly perinatal probiotic supplementation has been associated with altering the concentrations of TGF-ß1 and TGF-ß2 in human breast milk, thus increasing the immune quality of human breast milk.

IL-10 is an anti-inflammatory cytokine produced by monocytes, activated B cells, macrophages, mast cells, keratinocytes, dendritic cells and T cells (Moore *et al.* 2001; Asadullah *et al.* 2003). IL-10 antagonises synthesis of IL-12, TNF-α and IFN-γ (Pessi *et al.* 2000) and IL-10 stimulates proliferation and antibody production by B cells; including cooperation with TGF-β to stimulate IgA production by human B

cells and down-regulates IgE synthesis by enhancing class switching to IgG4 synthesis (Moore *et al.* .1993; Pessi *et al.* 2000). Also, IL-10 targets various cells involved in the allergic response and is a powerful inhibitor of pro-inflammatory cytokine production. IL-10 downregulates eosinophil survival and inhibits IgE-mediated activation of mast cells. IL-10 impairs DC maturation reducing their stimulatory capacity for induction of Th1 and Th2 cells. Furthermore, IL-10 also promotes IgG4 production in B cells, leading to a favourable ratio of IgG4 to IgE (Hawrylowicz and O'Garra 2005).

T-cell production of IL-10 is triggered at the time and site of allergen exposure (Urry *et al.* 2006). In humans, allergic individuals have a propensity for lower proportions of allergen-responsive IL-10-secreting $CD4^+$ T cells and more IL-4 secreting cells in their peripheral blood compared with their healthy non- allergic counterparts (Akdis *et al.* 2004). This suggests that a high IL-10 to IL-4 secreting T-cell ratio is important in the maintenance of immune homeostasis and prevention of inappropriate responses to allergens in healthy individuals. Interestingly alveolar macrophages from asthmatic patients secrete lower levels of IL-10 than normal non-asthmatic subjects (Urry *et al.* 2006). In addition, an inverse correlation between IL-10 levels and the severity of asthmatic disease has been described (Borish *et al.* 1996; Lim *et al.* 1998; Heaton *et al.* 2005).

1.4 Newborn immunity and the Th1/Th2 paradigm refined

Before one can accurately interpret the results of any intervention study assessing the immunomodulatory effects of perinatal probiotic supplementation on immunity; an appreciation for the progression of early neonatal immune development is warranted (Prescott and Nowak-Wegrzyn 2011). Neonatal organs undergo a dramatic transition at birth, from a sheltered intra-uterine existence to an environment where antigens are ubiquitous. This transition is countered by a gradual, age-dependent maturation of immunity in the post-utero environment. Given the limited exposure to antigens *in utero,* newborns rely strongly on innate immunity to maintain immune integrity and counter deleterious antigenic threats (Krishnan *et al.* 2003; Kenzel and Henneke 2006; Belderbos *et al.* 2009). Neonatal adaptive immunity is initially distinguished by a strong bias against production of Th1-polarizing cytokines and up-regulation of immune responses favouring the production of Th2 polarising

cytokines upon antigen recognition (Adkins *et al.* 2004; Chaouat *et al.* 2004). Although this Th1/Th2 bias renders the newborn prone to persistent Th2 mediated allergic responses and susceptible to microbial infection, the attenuation of Th1 immune responses in early life has been cited numerously as an evolutionary conserved precautionary mechanism originating *in utero* and extending to the *post-utero* environment to circumvent potentially harmful Th1 immune responses in early neonatal life. This phenomenon is supported by evidence that infection-induced production of the Th1 pro-inflammatory cytokines is associated with premature and pre-term delivery; and aberrant TNF-α production is a hallmark of miscarriage via apoptosis induction in placental and foetal cells (Vitoratos *et al.* 2006).

However, after birth there is an age-dependent maturation of the immune response and a gradual attenuation of the initial biased Th2 predisposition. This is concomitant with an increasing affinity towards maturation of antigen and/or microbial mediated Th1 immune responses and is influenced by prenatal and postnatal exposure to environmental microbial products that can activate innate immunity that might accelerate this maturation process (Bach 2002; Liu and Leung 2006; Ng *et al.* 2006). Therefore it is postulated that Th2-dominated immune responses reminiscent of the *in utero* environment undergo microbially-driven post-natal maturation, to circumvent development of Th2 mediate atopic disorder.

To provide a mechanistic insight into the immunomodulatory potential of probiotic bacteria in neonatal immunity; an appreciation for the threefold demands of neonatal immunity are paramount. These demands encompass:

(1) Protection against infection at the maternal-foetal interface (Barton 1984; McDonagh *et al.* 2004).

(2) Immune adaptation during the transition between the sterile intra-uterine environment to the antigen-rich post-utero environment due to microbial colonization of the skin and GIT (Karlsson *et al.* 2002; Marchini *et al.* 2005), and

(3) Avoidance of harmful alloimmune reactions between mother and foetus by inhibition of Th1 immune responses at the maternal-foetal interface (Makhseed *et al.* 2001).

1.4.1 Correlation between underlying cytokine predisposition and the onset of allergic disorder in infancy.

In newborns that develop the atopic phenotype clear developmental and early pre-symptomatic differences manifesting post-utero are already evident (See Table 1.4) when compared to healthy neonates at the same developmental stage. Early pre-symptomatic immunological imbalances in effector cytokine function are consistently reported and these are associated with imbalances in the quality and/or quantity of colonising intestinal probiotic bacteria. Key immunological features contributing to the increased neonatal risk of atopic disease include lower proliferative newborn T cell responses reduced cytotoxic T cell responses and dysregulated or attenuated cytokine production in response to microbial stimuli (Miles *et al.* 1996; Lewis 1998; Hartel *et al.* 2005). These symptoms are postulated to have a profound impact on the pathophysiology of atopic disorder. Epigenetic differences in immune function disrupting normal gene activation or silencing patterns required for an optimally functioning immune system are evident at birth between healthy and atopic neonates (Kuriakose and Miller 2010). Interestingly Th1 function is actively regulated by the foetus through epigenetic mechanisms that alter methylation patterns and gene expression of the IFN-γ promoter in effector $CD4^+$ T cells (Pfefferle *et al.* 2010).

The development of allergic disease is hypothesised to manifest with early pre-symptomatic differences in allergen-specific cytokine production (Beasley *et al.* 1998; Prescott *et al.* 1998; Prescott *et al.* 1999). Studies employing the stimulation of human mononuclear cells with the cytokine inducing lymphocyte mitogens phytohaemagluttin (PHA) and Staphylococcal enteroxin B (SEB) have provided invaluable insight into the immunology of atopic disorder. These studies have revealed that early pre-symptomatic differences exist in cytokine production ability by mitogen stimulated mononuclear cells isolated from neonates in whom atopy was and was not developing. PHA stimulated mononuclear cells isolated from atopic children, consolidate IL-4, IL-5 and IL-13 responses; and insufficiently upregulate IFN-γ responses between 6 months and one year of age compared to non-atopic counterparts (Tang *et al.* 1994; Prescott *et al.* 1999; Herberth *et al.* 2010). Numerous authors have demonstrated that infants who eventually express the atopic phenotype

by 12 months of age already present with significantly stronger Th2 T cell responses to allergens and mitogens by 6 months of age (Prescott *et al.* 1999). This immunological predisposition is concomitant with impaired Th1 IFN-α responses during the first months of life and persists in children who develop inhalant sensitisation by 6 years of age (Sudo *et al.* 1997). Furthermore, attenuated IFN-γ production is a hallmark of the immune response at birth in children with an increased risk of atopic sensitisation (Wilson 1991; Tang *et al.* 1994; Warner *et al.* 1994; Nurse *et al.* 1997; Campbell *et al.* 1999; Smart and Kemp 2001), concomitant with a failure to efficiently upregulate IFN-γ during infancy (Prescott 2003; Schaub *et al.* 2008; Smith *et al.* 2008; Tulic *et al.* 2011). These observations have been attributed to an underlying impairment of IL-12 production by neonatal T cells necessary for development of Th1 biased cells (Nilsson *et al.* 2004). Additionally a reduced capacity to produce IL-12 at birth is associated with a reduced number of IFN-γ producing cells associated with weaker allergen-specific Th1 responses and development of skin prick reactivity and atopic dermatitis during the first 2 years of life (Upham *et al.* 1999).

The postulate that the capacity to produce Th1 (typically IFN-γ), Th2 (typically IL-13) and regulatory cytokines IL-10 is diminished at birth in children at genetic risk of developing childhood allergic disease is now widely accepted (Holt *et al.* 1992; Tang *et al.* 1994; Warner *et al.* 1994; Prescott *et al.* 1998).

Lower proliferative responses and dysregulated or attenuated microbal mediated cytokine production by newborn T cells are key signature features contributing to the increased risk of atopic disease during infancy. These observations have been linked to imbalances in the quality and/or quantity of colonising GIT probiotic bacteria and are postulated to have a profound impact in the pathophysiology of childhood IgE-mediated atopic disorder (Hartel *et al.* 2005).

Cytokine production abnormalities seen in the atopic newborn support an arena for an increased propensity for the differentiation of Th2 biased T cell responses mediating increased peripheral blood IgE levels and eosinophilia during infancy (Prescott *et al.* 1999; van der Velden *et al.* 2001; Neaville *et al.* 2003). This suggests

that events regulating the expression of atopic disorder and allergen-specific T cell memory are operational pre- and/or post-natally when the antigen inexperienced immune system initially encounters innocuous allergens (Prescott *et al.* 1999; Baraldo *et al.* 2007). In summary cytokine imbalance marked by reduced Th1 secretion predispose an individual to the development of clinical atopic disease (Smart and Kemp 2002). This observation reinforces the rationale behind perinatal probiotic supplementation with Th1 inducing probiotics bacteria at the neonatal stage to circumvent the onset of allergy in clinically predisposed individuals.

Table 1.4 Pre-symptomatic differences manifesting post-utero in allergy-prone neonates

Innate Immunity	**Adaptive immunity**
The IL-12 p40-p35 heterodimer activates cell mediated Th1 immunity responses. Newborns display attenuated capacity to produce IL-12 due to a neonatal defect in p35 promoter nucleosome remodelling (Goriely *et al.* 2004).	Reduced overall capacity to produced cytokines due to reduced antigen presentation exhibited by neonatal dendritic cells and a high proportion of immature T cells with limited cytokine induction potential compared to low proportion of mature T cells with a greater cytokine induction potential.
Neonatal monocytes have reduced	Diminished capacity of infantile T cells to

expression of the MyD88 adaptor protein and are less responsive to LPS and bacterial lipopeptides signalling through TLR2 and TLR4 consequently an impaired capacity to produce TNF-α ensues.	produce IFN-γ due to selective attenuation of post-natal IFN-γ gene expression in T cells due to hypermethylation of CpG sites in the proximal promoter and reduced capacity to transcribe IFN-γ.
Neonatal dendritic cells exhibit reduced antigen presentation and lack the capacity to deliver polarising signals to T cells	

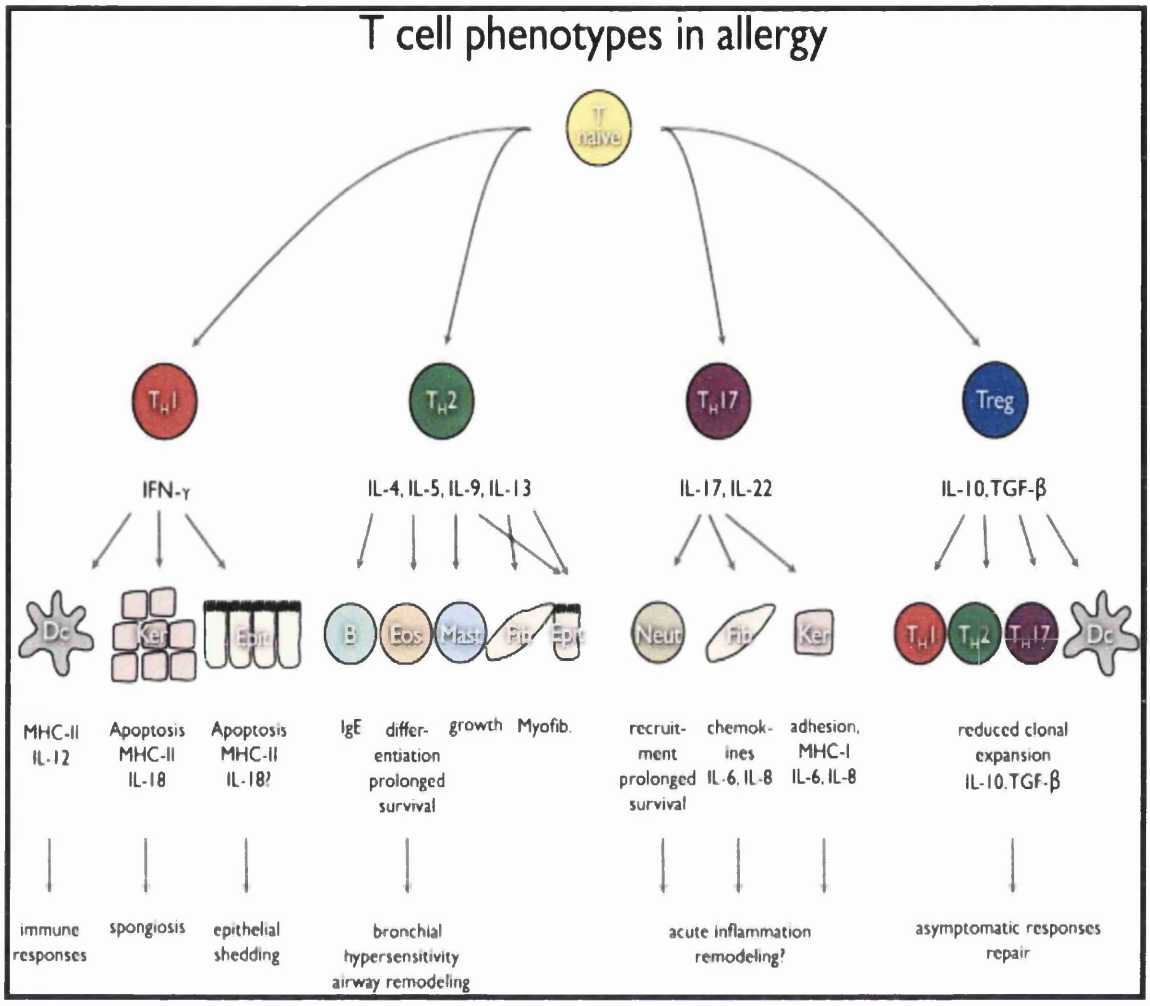


Figure 1.2. A schematic overview of T cell phenotypes in allergic disease and their role in orchestrating allergy development. Diagram adapted with permission from (Schmidt-Weber *et al.* 2007).

Th1, Th2, Th17, and Treg cells are characterized by cytokines, which mediate specific functions in different tissue cells such as the dendritic cells (DC), keratinocytes (Ker), epithelial cells (Epit), B cells (B), eosinophils (Eos), mast cells (Mas), fibroblasts (Fib), and neutrophils (Neut) (Schmidt-Weber *et al.* 2007). The continuous balance of T-cell subsets at the onset and during the course of disease determines the immunological features of the disorder.

The newborns inability to deviate from the *in utero* Th2 scheme of immunity is hypothesised to contribute towards atopic susceptibility. Additionally lower IFN-γ and IL-13 responses at birth are considered as indicators of a functionally immature immune system. The failure to efficiently upregulate IFN-γ and IL-12 is also critical

to the development of atopic sensitisation during infancy and this is associated with reduced number of IFN-γ producing cells (Upham *et al.* 2002; White *et al.* 2002; Prescott *et al.* 2003; Nilsson *et al.* 2004).

1.4.2 Atopic allergic march

The principal environmental risk factors for allergy development during the post natal period are early exposure to food allergens. This is associated with IgE-mediated allergic manifestations in the skin, GIT and respiratory tract (von Berg 2009). The manifestation of allergic disease in childhood generally follows a characteristic evolution known as the atopic allergic march, relating to the gradual development of the archetypal spectrum of atopic disorders persisting initially from early infancy to adolescence with atopic dermatitis representing the first clinical manifestation, followed by allergic symptoms of the upper or lower airways and IgE responses to alimentary or environmental allergens. IgE responses to cow's milk and hen's egg are usually primary immunological markers of atopy often substituted by IgE responses to indoor or outdoor allergens. Eczema and food allergy caused by food allergen sensitisation is often followed by aeroallergen sensitization, then a shift in disease burden to that of asthma and allergic rhinitis. However, the extent to which early life atopic sensitization sentences a child to a clinical history of allergic disease in later life is unclear (Kurukulaaratchy *et al.* 2005).

One of the key questions regarding the early life origins of allergic disease relates to the environmental factors that underlie the epidemic of allergy that has afflicted the developed world over recent decades.

1.5. The microfloral hypothesis and the role of the GIT microbiota in allergy pathogenesis

Since alterations in microbial exposure alone do not sufficiently explain the observed increase in allergic disease, attention has now focussed on the importance of the immunomodulatory role of commensal probiotic bacteria during gastrointestinal immune homeostasis.

The microbiota hypothesis states that perturbations in the gastrointestinal microbiota alter the symbiotic relationship between commensals and human immunity with disruptive effects on evolutionary conserved regulatory mechanisms necessary to

impart microbiota-mediated immunological tolerance towards allergens in the mucosa (Brandtzaeg 2009).

1.5.1 Differences in gastrointestinal commensal flora in atopic and non-atopic subjects

Variations in early intestinal colonization patterns have been implicated in the predisposition to allergic disease through effects on mucosal and systemic immune function. Recent studies comparing the microbial composition of the GIT microbiota in infants in whom atopy was and was not developing demonstrated that differences occurred: with anaerobic lactobacillus and bifidobacteria strains amongst the earliest and most predominant colonisers of the GIT in children showing resistance to the development of allergic disorder. Interestingly, infants manifesting with the allergic phenotype presented with more clostridia and staphylococci preceding the development of allergic disease concomitant with a reduced diversity of early faecal microbiota (Kalliomaki *et al.* 2001; Tang 2009). These studies linked postnatal differences in probiotic bacteria colonisation patterns in infancy (Alm *et al.* 1999) with pre-symptomatic immune dysregulatory differences pertaining to the development of clinical allergic disease (Bottcher *et al.* 2000; Kalliomäki *et al.* 2001; Prescott 2003) and logically led to the experimental application of probiotic bacteria to optimise the immunomodulatory potential of the intestinal microbiota. The principal goal of probiotic and allergy research is to, therefore, promote a favourable arena for enhanced development of innate, adaptive and oral tolerance immune mechanisms and to induce a probiotic mediated immunological equilibrium that is protective against the manifestation of IgE mediated atopic disorder in clinically predisposed atopic neonates (Prescott and Bjorksten 2007).

Interest in variations in the intestinal microbiota composition in relation to allergic disease during infancy was initiated by comparison of the faecal microbiota of Estonian versus Swedish 1 year old children. The intestinal microbiota of Estonian children had more lactobacilli and eubacteria but fewer clostridia; a microbiota reminiscent of that prevailing in infants of Western Europe in the 1960s. Changes in the intestinal microflora of infants in western industrialised countries were suggested (Sepp *et al.* 1997). This group then went onto compare the faecal microflora of allergic and non-allergic children from Estonia and Sweden. Allergic children from both countries had a similar microbiota which differed in composition to non-atopic

children: increased levels of aerobic microbes, particularly coliforms and *Staphylococcal aureus,* and decreased levels of anaerobic microbes, particularly lactobacilli, and bacteroides (Bjorksten *et al.* 1999). This group then undertook a prospective study, collecting faecal samples at 5 to 6 days and at 1, 3, 6 and 12 months and monitoring development of allergic disease until 2 years of age. Faecal samples were analysed using traditional microbial culture techniques. In comparison with infants who did not develop allergy (by skin prick test or clinical history), those who developed allergy were less often colonized with enterococci during the first month of life, and less often colonised with bifidobacteria during the first year of life and had higher counts of clostridia at 3 months of age (Bjorksten *et al.* 2001). The authors concluded that differences in the GIT microbial composition in infants who do and do not develop allergy are operational before the clinical manifestation of allergic disease. Prospective follow-up until 5 years of age has now been reported and allergy development remained associated with being less frequently colonised during the first 2 months of life with lactobacilli, bifidobacteria but also *Clostridium difficile* (Sjogren *et al.* 2009). Colonisation with *bifidobacterium* species was associated with exposure to higher levels of endotoxin and coming from a larger family.

Other analytical approaches frequently demonstrate similar differences in microbial flora composition in the GIT and allergic outcomes in early childhood irrespective of country: faecal levels of *C. difficile*-derived caproic acid was higher in Swedish allergic than in non-allergic children (Bottcher *et al.* 2000); gas liquid chromatography of bacterial cellular fatty acids and quantitative fluorescence in situ hybridization of bacterial cells demonstrated that as early as 3 weeks of age, Finnish children who developed atopic sensitisation had more clostridia and less bifidobacteria than children who remained non-atopic (Kalliomäki *et al.* 2001). A similar intestinal microbiota profile was also established in Japanese children presenting with atopic dermatitis (Watanabe, Narisawa *et al.* 2003). These studies highlighted the need for adopting methodologies beyond conventional bacterial cultivation techniques on Agar plates. An anthroposophic lifestyle inclusive of consumption of fermented vegetables, restricted use of antibiotics and vaccinations was also associated a more desirable intestinal microbiota composition and a lower incidence of allergic disease (Alm *et al.* 2002; Shreiner *et al.* 2008). Taken together, these studies highlight a relationship between subclinical intestinal microbial

exposure and allergic outcomes in later life. Antibiotic use in early life would clearly impact on this.

1.5.2. Antibiotic use

The attractiveness of the link between antibiotic use and allergic disease, lies in the data on national trends of antibiotic use versus the incidence of allergic disease, and in the impact of oral antibiotic use on the gastrointestinal microbiota (Shreiner *et al.* 2008). As discussed above, the intestinal microbial environment in infancy clearly has a pivotal role in the aetiology of atopic disorder. The use of antibiotics in the first year of life has been associated with increased risk of developing asthma, hayfever and eczema and allergic sensitisation in childhood, but the association with wheezing/asthma outcomes is the most robust relationship. Interestingly the likelihood of developing asthma was greater for antibiotic use in the first year of life than after the first year of life, or if never used (Wickens *et al.* 1999). Consequently early and repeated antibiotic usage has been implicated as a surrogate to identify infants who are most susceptible to development of atopic disorder in later life (Mullooly *et al.* 2007). However studies in which antibiotic use for lower respiratory tract infection was excluded showed no detrimental effect of antibiotic use in the first year of life on the development of a spectrum of atopic disorders including: asthma, allergic rhinitis or eczema (Illi *et al.* 2001; Celedon *et al.* 2002).

1.5.3 The Hygiene Hypothesis

The expression of the atopic phenotype appears to be synonymous with the industrialized environments of the affluent world. The hygiene hypothesis links a marked reduction in the level of microbial exposure and childhood infections, compared to that prevailing in less affluent regions as a causal factor in the rise in allergic disorders (Von Hertzen and Haahtela 2004). Increased use of antimicrobial medication, consumption of sterile food, reduced family size and improved or exaggerated hygiene and health care practices (See Table 1.5.3), typical of the western lifestyle during early childhood, is postulated to favour an arena for lower infection rates during childhood. This is concomitant with biased immune polarization toward a Th2 phenotype during postnatal immune maturation., and has led to a relative lack of microbial stimulation of the infant GALT (Rosalind and Bloomfield 2004).

At the immunological level the hygiene hypothesis proposes that the microbial environment interfaces with the innate immune system and modulates its ability to impart instructions to adaptive immune responses, most critically *in utero* and/or in early life (Vercelli 2006). The microbiota hypothesis substantiates notions introduced in the hygiene hypothesis and proposes that signals generated following microbial exposure modulate immunity towards ubiquitously occurring environmental allergens (Pan *et al.* 2010). The intestinal microbiota composition provides an important immunomodulatory inoculum for postnatal maturation of the immune system via induction of innate and adaptive immune mechanisms for programming Treg cells responses and non-inflammatory IgA responses. This is most relevant in infancy when postnatal immune programming is initiated (Prescott and Bjorksten 2007; Prescott *et al.* 2010)

The rationale behind the application of probiotics as a therapeutic strategy against IgE-mediated atopy stems from observations that prolonged prenatal and postnatal exposure to environmental microbes activates innate immunity in accord with the hygiene hypothesis (Strachan 1989). This regulates the Th2 bias of neonatal immunity by enhancing Th1-cell polarization, thus attenuating immune responses that support IgE mediated atopy (Bach 2002; Liu and Leung 2006).

However efforts to manipulate the Th2/Th1 equilibrium with probiotic bacteria should be conducted with caution, with the penultimate goal to induce a T regulatory cell response with low grade inflammation (Marschan *et al.* 2008); as opposed to an excessive Th1 immune response linked to the development of autoimmune disease. The introduction of artificial newborn feeding regimens and contemporary hygiene practices is postulated to encourage undesirable colonisation of the GIT. This is marked by immunological priming and IgE mediated clonal expansion of allergen specific Th2 lymphocytes and over excessive immunology towards allergens (Romagnani 2004). Aberrant Treg cell function in infants who subsequently develop allergic disease has been reported, along with the conjecture that immaturity of both Th1 and Treg compartments increase the risk of inappropriate persistence of a Th2 immunity in atopic individuals (Bousquet *et al.* 2004; Miller and Ho 2008; Smith *et al.* 2008; Martino and Prescott 2010).

Although the initial interpretation of the hygiene hypothesis was rather simplistic, its immunological basis has foundations in epidemiological and socio-economic data implying a causal link between changes in microbial load and allergic disease (Rosalind and Bloomfield 2004). Epidemiologic support for the hygiene hypothesis linked to the effects of intestinal microbiota microbial imbalances on immune development and the pathology that ensues in the absence of probiotic organisms of lactobacilli and bifidobacteria origin flourishing in the GIT (Bjorksten 2004). These strains are hypothesised to affect the quality of immune stimuli required for normal maturation of post-natal immunity to allergens, pathogens and non-pathogenic commensals (Strachan 1989; Vaarala 2003; Watanabe *et al.* 2003; Kabesch and Lauener 2004).

An alternative arm of the hygiene hypothesis suggests that the lower microbial burden has served to decrease activity of Treg cells. This notion arose from epidemiological observations highlighting that expression of the allergic phenotype is lower in prevalence and far less severe in regions of the world debilitated with a chronic persistence of helminth infections known to induce strong and persistent Th2 responses in concert with high production of suppressive cytokines (Romagnani 2004). Furthermore demonstration that exposure of a pregnant mother and newborn during the first year of life to microbial products released by farm animals, exerts an important protective effect against the development of allergy. This observation was attributed to the chronic stimulation of the TLRs, yielding further support for the hygiene hypothesis (Braun-Fahrlander *et al.* 2002). Microbial colonisation of the GIT is also linked with lifestyle and/or geographic factors, postulated to be an important determinant in the worldwide rise in allergic disorder as summarised in table below (Bjorksten 2005).

Table 1.5.3: Environmental risk factors for the development of food allergy

Measures to avoid or reduce microbial exposure	Time window when measure is likely to affect immune system	Effect on immunomodulatory organisms
Purification of household water supplies via chlorination.	Pregnancy, early childhood and during adult life	Reduces mycobacteria and other pathogenic microorganisms present in untreated water.
Cleaner home environments marked by better personal hygiene, increased use of household detergents and proper disposal of sewage waste water.	As above	Exposure to household pathogens and enteric organisms in sewage reduced.
Improved access to modern medicinal practices e.g. antibiotic therapy for infectious disease.	As above	Indiscriminate destructive action of antibiotics eradicates both probiotic microorganisms and infective microorganism.
Increased use of processed foods.	As above	Increases the growth of putrefying bacteria like clostridia in the intestinal tract.

Table 1.5.3.1: Incidental reductions in microbial exposure imposed by lifestyle

Incidental reduction in microbial exposure	Time window when measure is likely to affect immune system	Influence on immunomodulatory organisms
Lack of rural exposure or increasing urban dwelling	Early childhood	Reduction in exposure to environmental mycobacteria and enterobacteria
Less outdoor exposure to sunlight or fresh air	Early childhood	Less exposure to airborne microbes in the natural environment

1.5.4 The commensal microbiota and its establishment during infancy

Microbial communities are specified by the term "microbiota", defining a unique array of host specific micro-organisms that colonise distinct niches within the host according to their metabolic and survival needs. Processes involved in the establishment of the microbiota from neonate to adulthood are intricate and depend on bacterial succession as well as cross talk between bacteria and TLR-mediated immune recognition implemented by the host (Mackie *et al.* 1999). Approximately 100 billion to 1,000 billion beneficial bacteria per millilitre literally consume harmful bacteria and other pathogens (Barron 1999). Probiotics, in particular, form part of the resident commensal microflora community and have been implicated as enhancers of the immunomodulatory capacity of the intestinal microbiota (Crane 2002).

The newborn is rapidly colonised initially by bacteria from the surrounding environment of maternal origin following passage through the birth canal. Further colonisation commences immediately after birth (Adlerberth 1999), with aerobic and facultative anaerobic bacteria encompassing the coliforms, enterobacteria, lactobacilli, staphylococci and streptococci strains forming the principal colonising organisms (Kuhn *et al.* 1986; Cooperstock *et al.*1983). These strains are postulated to lower the oxidation reduction potential in the intestine allowing establishment of anaerobes such as bacteroides and bifidobacteria. The most common anaerobic genera in terms of concentration within the GIT are the strict anaerobes, forming the majority bacterial stakeholder residing at approximately 97% of the total inoculum and encompassing strains from the *Bifidobacterium, Lactobacillus, Bacteroides, Clostridium, Fusobacterium,* and *Eubacterium* genres. The (facultative anaerobes) form a minority bacterial population at only 3% with principal strains emerging from the corynebacteria and propionibacteria families, the Gram-negative enteric bacterial families including *E. coli, Veillonella* and *Salmonella* spp, the Gram-positive cocci including *Peptostreptococcus, Peptococcus, Enterococcus, Micrococcus, Staphylococcus* and *Streptococcus* and the fungal species *Candida albicans* are chief coloniser of the GIT (Noverr and Huffnagel 2004).

During infancy, the GIT develops a complex ecosystem stabilizing at approximately 10^{14} organisms with approximately 30-40 species believed to flourish. The composition gradually alters to resemble that of the adult, reaching densities of 10^{12} organisms per ml of luminal contents consisting of approximately 100 trillion microorganisms, from some 400 to 1000 different species (Simon and Gorbach 1984).

The GIT microbiota composition differs both cross-sectionally and longitudinally (Shreiner *et al.* 2008) with approximately 60% of the composition non-culturable employing contemporary techniques and only around 300-500 species currently culturable *in vitro* (Isolauri *et al.* 2001). Improvements to PCR and genome sequencing techniques permit further characterisation of the intestinal microbiota and discovery of strains with potential probiotic effects.

The development of the microbial community plays a pivotal role in regulating the maturation of the newborn immune system. Gastrointestinal microbial imbalances

occurring early in life are postulated to lead to the induction of insufficient tolergenic mechanism supporting the development of immune dysregulated disorders. Therefore the rationale behind perinatal administration of probiotic bacteria is to influence favourable colonisation of the newborn GIT.

From culture based data, the mouth harbours a complex microbiota consisting of facultative and strict anaerobes, including streptococci, bacteroides, lactobacilli, and yeasts (Sekirov *et al.* 2010). The mammalian lower intestine contains an extremely diverse and dense flora of commensal bacteria not normally pathogenic in an immunocompetent host (Noverr and Huffnagel 2004).

Table 1.5.4 Distribution of the microflora within the GIT

Stomach and Duodenum	Principally lactobacilli and streptococci colonise this region. Harbours relatively low bacterial numbers: $< 10^5$ bacterial cells per gram of contents. Phasic propulsive motor activity in synergy with the actions of acid, bile and pancreatic secretions impedes stable colonisation of the lumen,
Jejunum and ileum	Bacterial numbers progressively increase from approximately 10^4 cells in the Jejunum to 10^7 cells per gram of contents in the distal ileum.
Large intestine	Heavily populated by anaerobes 10^{12} cells per gram of luminal contents.

Infant feeding regimens play a crucial role in establishing the commensal microbiota of the GIT immediately after birth. Major changes in gut microbiota composition

occur during breast feeding, weaning, and the transition to solid foods (Cooperstock *et al.* 1983). Penders *et al.* (2006) evaluated a broad range of external influences on acquisition and composition of the gut microbiota in infancy.

After analysis of 1032 faecal samples from babies aged 1 month of age the most important determinants of infantile gut microbiota composition was proposed to be:

1. Mode of delivery.
2. Birth environment.
3. Gestational age.
4. Mode of feeding, early life antimicrobials and other medicinal usage.
5. Early life exposure to infection.
6. Infant hospitalization.

Thus, the establishment and maintenance of a probiotic optimised GIT is determined by factors affecting early microbial populations a complex process influenced by microbe-microbe and microbe-host interactions (Mackie *et al.* 1999).

The first observations that significant differences existed in the microbiota composition in breast-fed versus bottle-fed infants were made a century ago. The microbiota of breast-fed infants was dominated by LAB and bifidobacteria species, while formula-fed infants harboured a more complex and diverse adult-like microbiota characterised by a mixture of anaerobic and aerobic bacteria with enterobacteria, lactobacilli, bacteroides, clostridia, *E. coli*, *C. difficile*, bifidobacteria and streptococci predominating (Penders *et al.* 2006). The predominance of

bifidobacteria associated with exclusive breast feeding is postulated to offer protection against gastroenteritis. Scientific interest in bifidobacteria was promoted by the observation that breast-fed infants were less susceptible to infections and the allergic phenotype in later life than bottle-fed babies. It was postulated that bifidobacteria in the colon might play a role in the newborns resistance to infection (Yasui *et al.* 1989). More recent studies of infant GIT microbiota have failed to confirm exuberant colonisation with bifidobacteria in exclusively breast fed infants, a change attributed to changes in maternal diet or obstetric practices (Lundequist *et al.* 1985; Hall *et al.* 1990).

Breast milk is rich in biogenic prebiotics oligosaccharides (growth substrate for probiotics), probiotic micro-organisms such as bifidobacteria are postulated to be present in human breast milk (Martin *et al.*2003; Gueimonde *et al.* 2007). An intrinsic evolutionarily conserved mechanism operating in human breast-milk appears to support and drive the colonisation of an infant gut microbiota rich in a diverse array of beneficial probiotic microorganisms. The occurrence of oligosaccharides, like fructo-oligosaccharide (FOS) and galacto-oligosaccharide (GOS) renowned for their ability to influence and support growth and survival of LAB and bifidobacteria populations (Newburg 2009), underscores this premise. Upon introduction of solid food, the intestinal microbiota increasingly resembles that of adults, reaching a stable microbial ecosystem in which anaerobes predominate.

Mode of delivery also impacts on the composition of the infant microbiota. Numerous studies of microbiota development have shown major differences in culturable microbiota in vaginally versus caesarean section delivered newborns. Infants born by caesarean section harboured a paucity of bifidobacteria and bacteroides, concomitant with frequent colonisation by *C. difficile,* compared with vaginally delivered infants. Hospitalisation and prematurity was also associated with elevated *C. difficile* counts. In preterm infants, treatment with antibiotics or being nursed in an incubator significantly reduced early colonisation with lactobacilli and bifidobacteria (Hall *et al.* 1990). Improved maturation of humoral immune mechanisms such as elevated circulating IgA and IgM-secreting cells have been observed at 6 month of age in vaginally delivered infants who tended to harbour more *Bacteroides fragilis* and *Bifidobacterium* species than caesarean delivered infants. Term infants born vaginally

at home who were breastfed exclusively also seemed to have the most "beneficial" gut microbiota (Gronlund *et al.* 2000).

1.5.5 The intestinal microbiota and the immune system

The gut microbiota is exposed to host physiological systems and coexists peacefully within the host, irrespective of immunogenicity. This symbiotic host-microbe relationship has co-evolved in the presence of the intestinal microbiota and the gastrointestinal immune system (Sekirov *et al.* 2010) and is key during establishment of normal gut barrier functions, regulation of systemic and local immune responsiveness and hyporesponsiveness (oral tolerance) to food and microorganism derived antigens (Cebra 1999; Nagler-Anderson 2001; Noverr and Huffnagle 2004; Brandtzaeg 2009; Chung and Kasper 2010). The gut microbiota is postulated to stimulate the immune system via induction of a subtle mode of intestinal inflammation regulated by TLR-mediated immune recognition of probiotic bacteria. This contrasts to clinical mucosal inflammatory response mediated upon immune recognition of pathogenic organisms (O'Farrelly 1998; Viljanen *et al.* 2005; Marschan *et al.* 2008).

Intestinal commensals play key roles in gastrointestinal health (See Table 1.5.5) by assimilating indigestible nutrients of human metabolism, competitively excluding harmful bacteria from gaining a foothold and facilitating processes of digestion, absorption and B vitamin and enzymes synthesis (Barron 1999; Isolauri *et al.* 2001).

TLR-mediated interactions between the immune system and the gut microbiota occurring at the GALT do not cause overt inflammation in healthy individuals (von Hertzen and Haahtela 2006). Normal development of the immune system depends on tolerogenic symbiosis with the microbial ecosystem, and is important in mediating memory mechanisms of systemic immunity (Guarner and Malagelada 2003[a]; Guarner and Malagelada 2003[b]). Probiotic bacteria, in particular are noted for their immunomodulatory influence on innate and adaptive immune mechanism that contribute to an immune microenvironment in which non-pathogenic tolerogenic responses to food antigens and allergens occur (Hooper and Macpherson 2010).

A diverse range of haematopoietic cells are found within the GIT including lymphocytes, macrophages and DCs. These cells participate in the overall immune response and the number of intraepithelial lymphocytes (IELs) is approximated to be more than half the T cell repertoire estimated for peripheral lymphoid organs (Noverr and Huffnagel 2004). Greater than 70% of all haematopoietic cells of the host are located in the intestine to combat the challenge presented by environmental antigenic load. Undeveloped mucosal immunity is cited as the reason for higher vulnerability to post-natal infections and sensitisation to dietary antigens during infancy (Kalliomaki *et al.* 2003). Although mucosal protection marked by locally produced secretory IgA is quantitatively and functionally defective for a variable period after birth; probiotic lactobacilli and bifidobacteria of intestinal origin have been demonstrated to enhance IgA responses (Kalliomaki *et al.* 2003).

Stimuli provided by commensal bacteria are crucial for the development of a fully functional and balanced immune system, including homing of B and T cells to the lamina propria and expansion and maturation of IgA plasmocytes and IgA production (Isolauri *et al.* 2001). During infancy, principal components of the gut barrier are immature rendering the intestinal surface relatively permeable, thus enhancing proneness to inflammatory responses. Disturbed microbiota succession is linked to the risk of developing infectious, inflammatory and allergic diseases in later life. Experimental and clinical follow-up studies suggest that constant stimulation by the intestinal microbiota strongly outweighs the importance of occasional infections in providing the essential immunological provocation necessary for maturation of gut barrier functions and innate and adaptive immunity (Isolauri *et al.* 2009).

1.5.6 The effect of probiotics on the allergy-associated immune response and T cell responses

An impressive body of *in vitro* data evidence exists detailing the role of probiotics in favourably modulating the intrinsic Th2 skewed nature of allergen specific T cell responses in a Th1 dependent manner (Cross *et al.* 2001; Niers *et al.* 2005; Flinterman *et al.* 2007). Nonetheless mechanisms by which probiotics regulate allergic responses *in vivo* remain to be fully characterised.

The anti-allergy strain specific function of bifidobacteria and lactobacilli is well documented both *in vitro* and *in vivo* and is postulated to occur via the immunomodulatory action of Th1 cytokines, (Isolauri *et al.* 2000; Paganelli *et al.* 2002) TGF-β and intestinal IgA upon induced immune recognition during the Th2 allergy driving phase of the disorder (Kaila *et al.* 1992; Kaila *et al.* 1995; Isolauri *et al.* 1995; Miettinen *et al.* 1998; Fukushima *et al.* 1999; Kirjavainen *et al.* 1999; Phuapradit *et al.* 1999; Hessle *et al.* 1999; Christensen *et al.* 2002; Ichikawa *et al.* 2009). Numerous authors have demonstrated that stimulation of human mononuclear cells with probiotic strains can induce a strain specific production of a range of inflammatory cytokines (Isolauri *et al.* 2001). Interestingly probiotic induced IL-12 and IFN-γ production by mononuclear cells has been demonstrated to not only induce Th1 differentiation, but also shift *in vitro* allergen-specific Th2 responses towards a Th1-polarised phenotype. This effect has been replicated *in vitro* with microbial CpG-containing oligodeoxynucleotides mixed to, or conjugated with allergens, resulting in production of high quantities of Th1-polarising cytokines by intestinal macrophages (Parronchi *et al.* 1999; Tighe *et al.* 2000)

The Th1 cytokine inductive capacities of probiotic bacteria is postulated to antagonise Th2 cytokine release from mitogen stimulated peripheral blood mononuclear cells (PBMCs), a property with anticipated benefits for the management of atopy *in vivo* (Pochard *et al.* 2002). In support of this induction of Th2 cytokines by PBMCs stimulated with the superantigen e.g. SEB is inhibited by prior exposure to a four strain mix of *Lactobacillus plantarum*, *Lactococcus lactis*, *Lactobacillus casei* and *Lactobacillus rhamnosus*. This inhibitory effect was not observed in the presence of the Gram-negative bacterium *E. coli* TG1 and was dose dependent with higher strain doses leading to more pronounced reduction of IL-4 and IL-5. Interestingly bacterial viability did not impact upon cytokine induction ability as heat killed bacteria performed equally as well as exponentially growing strains (Pierre 2002).

In another study, production of IL-4, IL-5 and IL-13 by PBMCs from allergic patients sensitised to aeroallergens was attenuated significantly when PBMCs from these patients were incubated with LAB and the relevant aeroallergen *in vitro* (Pierre 2002). This study further highlighted the role of probiotic bacteria in limiting allergic

responses, and modulating Th1/Th2 activity *in vitro*. This has encouraged support for the application of perinatal probiotic supplementation as a strategy to circumvent atopic disorder later in life (Cross *et al.* 2001). The net effect of probiotic-mediated inflammatory cytokine production *in vivo* is postulated to be the inhibition of IgE secretion from plasma cells orchestrated by the inhibitory effect of inflammatory cytokines on Th2 responses (Sudo 1997; Heller 2003; Osbourne 2008; Ichikawa *et al.* 2009).

1.5.7 Effects of probiotics on T cell responses

The TLR-mediated recognition of Th1 cytokine inducing probiotic bacteria is postulated to alter the cytokine microenvironment induced during a Th2 atopic response and attenuate upregulation of a Th2 mediated allergic response (Prescott and Dunstan 2005). Some probiotic strains have a greater *in vitro* capacity to stimulate Th1 cytokine production; while others induce Th2 or mixed, Th1/Th2 responses (Tang 2009). (Christensen *et al.* 2002) highlighted the specific immunomodulatory qualities of probiotic bacteria. *Lactobacillus casei* for instance is a strong inducer of TNF-α, IL-12, and IL-6 whereas *Lactobacillus reuteri* is a poor IL-12 inducer. Interestingly, some bifidobacteria and lactobacilli strains have inhibitory or opposing effects on regulatory function and differentially modify each other's effects. For instance (Christensen *et al.* 2002) demonstrated that *Lactobacillus reuteri* specifically inhibits *Lactobacillus casei*-induced DC cytokine production, and up-regulation of co-stimulatory markers; and *Bifidobacterium lactis* downregulates TGF-β-production.

Bifidobacteria and lactobacilli have been postulated to promote regulatory responses but mechanisms for doing so are variable. Bifidobacteria induce production of the regulatory cytokine, IL-10 by both myeloid and plasmacytoid DCs and down regulate expression of co-stimulatory molecules CD80 and CD40 on DCs. Down-regulation of IFN-γ appears to be IL-10-dependent. Contrastingly, specific *Lactobacillus* species induce Treg cells by generating semi-mature DCs with increased expression of co-stimulatory molecules and lower levels of pro-inflammatory cytokines concomitant with a down regulation or negligible effects upon IL-10 production (Hart *et al.* 2004).

In another study, *Lactobacillus paracasei*, strongly inhibited $CD4^+$ T cell proliferation and production of the IFN-γ and IL-4, IL-5, but produced the regulatory cytokines IL-10 and TGF-β in a dose-dependent manner (von der Weid *et al.* 2001). Similarly *Lactobacillus rhamnosus* induced T-cell hyporesponsiveness to CD3/CD28 stimulation and reduced production of IL-2, IL-4 and IL-10 both *in vitro* and following *in vivo* administration (Braat *et al.* 2004). Probiotic bacteria predominantly modulate DC and T-regulatory cell activity, although effects on Th1 or Th2 immune pathways (Tang 2009).

In vitro and clinical studies with probiotics suggest that *Lactobacillus rhamnosus* GG (LGG) attenuates Th2 immune responses by promoting Th1 responses. The cytokine profile induced by LGG-stimulated PBMCs is characterised by increased IFN-γ and IL-12 production, no change in IL-4 production and a modest increase in IL-10 production (Miettinen *et al.* 1998).
Comparable Th1-promoting effects have been demonstrated *in vivo* with increased production of IFN-γ in infants with cow's milk allergy and eczema given an LGG-containing dietary supplement (Pohjavuori *et al.* 2004). The increase in IFN-γ levels from mononuclear cells in response to probiotics could offer a mechanistic explanation for the possible clinical benefits of probiotics in controlling the manifestation of atopic diseases (Vaarala 2003). However, a cautionary note is proffered by the cow's milk allergy study as in a parallel study group, a consortium of four probiotics that included LGG had no effect on IFN-γ production and increased IL-4 production indicating that different and potentially antagonistic immune responses can be mediated by individual or mixtures of probiotic strains. In support of this premise, in animal models of Th1 mediated autoimmune arthritis, some probiotics strains inhibited Th1 immune responses providing alleviating effects on disease progression whilst others exacerbated arthritic pathology by potentiating Th1 immune responses within the already Th1 biased disease state (Lamacchia *et al.* 2010).

The observations that elevated levels of the transcription factors NF-κB and STAT6 occurred subsequent to stimulation of human monocytes with *Lactobacillus rhamnosus* cell wall components (Miettinen *et al.* 2000) has allowed researchers to deduce the signalling pathways employed by probiotic bacteria to induce a cytokine response. Toll-like receptor signalling in small intestinal epithelium promotes B-cell

recruitment and IgA production in the lamina propria (Guarner and Khan 2008). Interestingly, in a mouse model constant activation of TLR4 in the intestinal epithelium did not lead to unbalanced inflammation but rather elevated production of secretory IgA, highlighting an increase in gut barrier function in response to constant TLR4 stimulation (Shang *et al.* 2008).

Table 1.5.5: Summary of the immunologic and non-immunologic health benefits of a probiotically optimized GIT in terms of probiotic host interaction and symbiosis between microbiota. As reviewed by (Guarner and Khan 2008).

Immunological benefits	**Non-immunological benefits**
Activation of local macrophages to increase antigen presentation to B lymphocytes and increase secretory (IgA) production locally and systemically	Digest food, fecal matter and compete for nutrients with pathogens.
Modulation of Th1/Th2 and Th3 cytokine profiles	Alter local pH exerting an unfavourable local environment for pathogens

Induction of hyporesponsiveness to food antigens	Produce bacteriocins to inhibit pathogens
	Scavenge superoxide radicals
	Enhance intestinal barrier function and integrity and compete for adhesion sites with pathogens.
	Stimulate epithelial mucin production
	Exhibit enzymatic activity e.g. (β-glucosidae)

1.5.8 The immunomodulatory effects of probiotic bacteria in germ free mice

The importance of probiotic bacteria in immunity has been demonstrated by studying the pathology that ensues in GF mice, in the absence of the immunomodulatory presence of probiotics bacteria. In GF mice the establishment of the lymphocyte repertoire is impaired and mice present with multiple maturational defects linked to the absence of immunomodulatory signals provided by the intestinal microbiota, such as an inadequately colonized GIT, and reduced size of extra-intestinal lymphoid organs like the spleen, and thymus.

Interestingly studies in animal models indicate that the induction of IL-12 *in vivo* might reduce baseline serum IgE levels thereby improving the severity of allergic responses. From a mouse model using *in situ* analysis of Peyer's patches following oral administration of FITC-labelled *Lactobacillus paracasei* it was reported that (i) IgE production was reduced *in vivo*; (ii) *Lactobacillus paracasei* interacted with CD11b-positive cells and induced IL-12 mRNA expression in the Peyer's patch; and (ii) blood IL-12 levels increased transiently 10 hours after administration (Ichikawa *et al.* 2009).

In the absence of immunomodulatory signals provided upon recognition of MAMPS; cellular development of the lymphocyte compartment is severely compromised in GF animals, with impaired expansion of the CD4 T-cell repertoire representing a signature immunodeficiency. This phenomenon is postulated to occur in a cytokine dependent manner, with GF animals exhibiting systemic skewing towards an allergy supporting IL-4 mediated Th2 cytokine profile compared with conventionally colonized mice, which up-regulate a more balanced Th1/Th2 profile (Sekirov *et al.*

2010; McLoughlin and Mills 2011). Interestingly (Mazmanian *et al.* 2005) demonstrated that colonization of GF mice with the commensal organism *Bacteroides fragilis* alone was sufficient to promote a Th1 response and correct the Th1/Th2 imbalance seen in GF mice; in a signal transducer and activator of transcription 6 signalling (STAT-6) dependent manner in DCs that promoted IL-12–dependent expansion of Th1 cells, proliferation of $CD4^+$ T cells, and restoration of lymphocyte development (Falk *et al.* 1998). These observations in GF animals lend support for the rationale behind perinatal probiotic supplementation of the neonate. The maturational defects observed in germ free mice are summarised in Table 1.5.8

Table 1.5.8: Immunological defects in germ free mice due to a lack of an intestinal microbiota

Impaired macrophage chemotaxis and phagocytic activity. Fewer spleen-derived macrophage precursors with reduced expression of MHC class II (Starling and Balish 1981; Nicaise *et al.* 1998).
Decreased size of Peyer's patches and mesenteric lymph nodes lack of germinal centres, the site of B-cell proliferation and affinity maturation plasma cells production (Shroff *et al.* 1995)
Imunoglobulin class profile greatly altered with IgG concentrations much lower than in conventionally reared mice, with little or no production of IgA or antibody diversification (Wostmann 1981).
Fewer intestinal T cell and cytotoxic T cell populations (Umesaki *et al.* 1999)

1.6. Probiotics

1.6.1 Probiotics and health

Currently numerous strains of probiotic origin are commonly employed in the nutrition industry by virtue of their desirable metabolic and immunomodulatory properties that impact positively upon the integrity and functionality of the gastrointestinal and immune systems.

The existence of mutualism between probiotic bacteria and the host has long been appreciated with probiotics colonising a nutrient rich habitat, with a stable

temperature and pH to salvage energy from assimilation of otherwise indigestible GIT dietary constituents such as cellulose. This symbiotic benefit is postulated to have lasting longevity from foetus to adulthood. From a non-immunologic perspective, the dense intestinal microbiota concentration hinders pathogenic bacteria from adopting a facultative intracellular existence. Numerous studies have indicated that probiotics are capable of modulating the composition and/or activity of the intestinal microbiota, which in turn is expected to influence immune responses. The most commonly employed probiotic bacteria are bifidobacteria and lactobacilli (Isolauri *et al.* 2001).

Probiotics are typically employed as dietary supplements and have been used extensively for preservation of food via fermentation (Guarner and Khan 2008). The most common forms for probiotics are dairy products and probiotic-fortified foods containing the bacteria in freeze-dried form. Although many over-the-counter products deliver in the range of $1\text{-}10\times10^9$ colony forming units (CFU)/dose, some strains appear to be efficacious at lower levels, while some require substantially more. For instance, *Bifidobacterium infantis* has been demonstrated to be effective in alleviating the symptoms of inflammatory bowel disease at 1×10^9 CFU/day (Whorwell *et al.* 2006), whereas studies with VSL#3 (a cocktail of eight LAB strains including mixture of 1 strain of *Streptococcus thermophilus*, four Lactobacillus spp, and three Bifidobacterium spp. Strains) have used sachets with $3\text{-}4.5\times10^9$ CFU (Huynh *et al.* 2009). Therefore the general dose required for probiotics dosage is variable and should be based on human studies showing a health benefit (Guarner and Khan 2008).

Despite the influx of probiotics products to the supermarket shelves and increasing inclusion in dairy products, a cloud of mystery surrounds the potential of their immunomodulatory attributes and the mode by which they enhance immune integrity. A probiotic optimised GIT in healthy newborns is associated with maturation of humoral immune mechanisms, particularly of circulating IgA and IgM secreting cells, reflecting the dependency of the regulation of the mucosal immune response on the normal gut microbiota (Gronlund *et al.* 2000).

From an immunological and gastroenterological standpoint there are valid reasons for anticipating health benefits of probiotic supplementation. However there is a paucity of convincing scientific evidence recommending probiotics as part of a standard therapy in the setting of allergy disorder (Prescott and Bjorksten 2007). Furthermore, despite several studies indicating immunomodulatory benefits of probiotic supplementation during prevention of allergic disorder, a recent Cochrane systematic review concluded that, "upon pooling results for different probiotic strains used in clinical trials probiotics are not effective for the treatment of allergic disorder or atopic sensitization" (Osborn and Sinn 2007).

1.6.2 Perinatal supplementation with probiotics and prevention of allergic disease: clinical outcomes

In utero persistence of the Th2 mediated immune microenvironment is important for successful pregnancy to antagonise upregulation of toxic autoimmune Th1 responses deleterious to placental integrity (Makhseed *et al.* 2001). In healthy newborns the probiotic rich microbiota is postulated to act on regulatory T cells to shift the balance of immunity from over reliance upon the default *in utero* Th2 pathway; towards maturation of Th1, Th17, tolerogenic and non-inflammatory IgA mediated pathways. This shift occurs independently of the neonatal propensity to support immune responses of a Th2 mediated discourse in health neonates but is impaired in children presenting with atopy (Prescott *et al.* 1999).

The optimal timing, be it during pregnancy, postnatally or perinatally at which probiotic supplementation is most efficacious, and the mechanisms underlying their role in preventing atopy remains to be resolved. Nonetheless a window of opportunity for perinatal probiotic supplementation is postulated to exist both pre and postnatally when the newborn is most amenable to a favourable immunomodulatory contribution provided by intestinal probiotic bacteria Furrie (2005).This contribution is necessary to circumvent over reliance upon Th2 dominated neonatal immunity, a driver of atopic immune responses. Thus the quality of immune education provided by intestinal microbiota during infancy is vital for normal immune development (Bjorksten *et al.* 2001).

There are now numerous prenatal, perinatal and post-natal probiotic intervention studies: Features common to all perinatal probiotic intervention studies for the prevention of allergy were twofold: (1) All included participants with **at least** one family member expressing a phenotype of atopic disease i.e. at least one first-degree relative (or partner) with atopic eczema, allergic rhinitis, or asthma; and (2) At least one principal measurement of allergic outcome (for example allergen specific IgE data measured in plasma or by skin prick test (SPT) (See Table 1.6.1).

The landmark study by the Finnish group led by Professor Erika Isolauri, evaluated the allergy preventive effect of *Lactobacillus* GG supplementation on atopic disease among infants at high risk for atopic disorder using a perinatal double-blind, randomised placebo-controlled trial (Kalliomaki *et al.* 2001). *Lactobacillus* GG was administered to mothers daily for 2 to 4 weeks before expected delivery, and to their infants from birth to 6 months of age, and breastfeeding mothers. Of the 83% of neonates who completed the study to two years of age, 46% in placebo group had eczema but only 23% had eczema in the probiotic group. Probiotics had no effect on severity of atopic dermatitis. No differences appeared in specific IgE levels or SPT results (Kalliomaki *et al.* 2001). In the follow up study assessing the persistence of 'atopic' eczema protection, the difference in atopic eczema prevalence between the two groups was retained (Kalliomaki *et al.* 2003). However the probiotic group tended to have a greater proportion of children with symptoms of rhinitis/wheezing. Similarly to the previous study, no difference in atopic sensitisation was observed as determined by SPT positivity, since IgE-sensitisation did not differ between probiotic and placebo groups. In the most recent follow up study from this group, the cumulative risk for developing eczema in the first 7 years was significantly lower in the probiotic versus the placebo group but more children in the probiotic group had rhinitis (Kalliomaki *et al.* 2007).

In another study of LGG supplementation (1×10^{10} CFU/day to the pregnant women from 4 – 6 weeks before delivery and after birth to breast feeding mothers for 3 months and then to infants for another 3 months) there was no difference in the cumulative incidence to eczema to 2 years or the severity of disease. This was concomitant with no difference in total IgE concentrations or frequency of sensitisation observed in the placebo versus probiotic group. Worryingly probiotic

supplementation exacerbated incidences of childhood recurrent episodes of wheezing bronchitis (Kopp *et al.* 2008).

Interestingly, (Abrahamsson *et al.* 2007) discovered perinatal supplementation with *Lactobacillus reuteri* (1 x 10^8 CFU/daily) or placebo from 36 weeks of pregnancy until delivery and then to neonate until 12 months of age was associated with less IgE-associated eczema at age 2 years. Although the results were only significant at 2 years of age, the expression of eczema was consistently lower in prevalence in the probiotic group. In contrast to the (Kopp *et al.* 2008) study, the cumulative incidence of wheeze was similar at 2 years.

In a randomised double blind placebo controlled (RDBPC) perinatal study involving n =1223 recruits (Kukkonen *et al.* 2007) studied the effects of a consortium of probiotic strains: *Lactobacillus rhamnosus GG* (5 x 10^9 CFU); *Lactobacillus rhamnosus* LC705 (5 x 10^9 CFU); *B. breve* (2 x 10^8 CFU); *Propionibacterium freudenreichii* (2 x 10^9 CFU) strains on the cumulative incidence of food allergy, eczema, asthma and allergic rhinitis and IgE-associated disease over the first 2 years of life. Pregnant women took 2 capsules per day from 2 to 4 weeks before delivery and then the baby was administered strains from 1 capsule per day from birth to 6 months of age. Infants were also administered prebiotic galacto-oligosaccharides daily with (n =461) probiotic and (n=464) placebo completing the study to 2 years of age. No effect on the cumulative incidence of any allergic disease was discovered, but IgE associated disease tended to be less frequent in probiotic group with eczema and atopic eczema less likely in probiotic group. Similar to previous studies no effect on sensitisation at 6 or 24 months was observed. After adjustment for confounders, IgE-associated disease, eczema and atopic eczema was significantly lower in probiotic group.

In the follow up study Kuitunen *et al.*(2009) assessed the cumulative incidence of allergic and IgE-mediated allergic disease until 5 years of age. Again no difference in allergic or IgE-associated allergic disease, frequency of eczema, IgE-associated eczema, asthma, allergic rhinitis or atopic sensitisation was discovered. Despite caesarean-section deliveries being associated with a less favourable microbial colonisation of the newborn GIT (Biasucci *et al.* 2008), it was interesting to note that

caesarean-delivered children supplemented with probiotics in this study had significantly fewer IgE-associated allergic diseases, particularly eczema, and less IgE sensitisation.

In another RDBPC study, (Kim *et al.* 2009), employed a consortium of *Bifidobacterium bifidum*, *Bifidobacterium lactis* and *Lactobacillus acidophilus* and daily administered 1.6 x 10^9 CFU of the consortium to pregnant women, 8 weeks before expected delivery until 3 months after delivery. They also administered the consortium to the newborns either breast-fed or formula. Interestingly the prevalence of eczema in the probiotic group was significantly reduced in infants at 12 months of age, despite total IgE levels and frequency of atopic sensitisation being comparable between their consortium and placebo groups.

(Niers *et al.* 2009) studied maternal supplementation during the last 6 weeks of pregnancy and then postnatally to the baby for 12 months with 3 x 10^9 CFU/day of a consortium of *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus lactis* (1 x 10^9 of each organism). Interestingly, supplementation was associated with reduced prevalence in parentally reported eczema reduced in the probiotic group; subsequently confirmed following GP consultation. No differences in the groups for wheezing, cough, rhinitis and total IgE were found.

A study undertaken in New Zealand used a strategy of supplementation with 6 x 10^9 CFU/day of *Lactobacillus rhamnosus* HN001 (n= 34) or 9 x 10^9 CFU/day *Bifidobacterium lactis* HN019 (n = 35) or a placebo (n = 36) to the mother from 35 weeks of pregnancy to 6 months after delivery in lactating women. Their offspring received the same from around birth to 2 years of age (Wickens *et al.* 2008). Supplementation with L*actobacillus rhamnosus* but not *B. lactis* was associated with significantly reduced risk of eczema by 2 years of age and reduction SCORAD. Neither probiotic affected likelihood of having a positive SPT but those taking *Lactobacillus rhamnosus* presented with significantly reduced IgE-associated eczema.

In a Prenatal study (Boyle *et al.* 2008) investigated the role of probiotic supplementation in protection against the development of eczema. The authors

administered 1.8×10^{10} CFU of the probiotic *Lactobacillus rhamnosus* strain (LGG) daily for 7 days in a randomized controlled trial, and measured foetal immune responses *ex vivo*. CBMC samples were harvested from infants of 73 women with a history of doctor-diagnosed asthma, allergic rhinitis, food allergy or atopic eczema in themselves, their partner or a previous child, and cultured them with heat-killed LGG, ovalbumin (OVA) or without stimulus. Interestingly LGG pre-treatment of pregnant women did not influence CD4 T cell proliferation, forkhead box P3 expression, DC phenotype or cytokine secretion in CBMCs cultured with heat-killed LGG or OVA suggesting that prenatal probiotic supplementation failed to influence fetal antigen-specific immunity in a way that could be protective against eczema development.

In a postnatal probiotic supplementation study in which *Lactobacillus acidophilus* probiotic strain administered for the first 6 months (Taylor *et al.* 2007) worryingly reported that increased risk of allergen sensitization at 1 year of age was synonymous with supplementation. The follow up study assessed effects on subsequent allergic outcomes in 153 children from the initial prevention cohort at 2.5 years of age, and discovered that postnatal probiotic supplementation did not attenuate the risk of dermatitis at 2.5 years compared with that in placebo group. There was no significant reduction other allergic diseases or allergen sensitization. Interestingly children in the probiotic group had fewer gastrointestinal infections in the preceding 18 months.

In another study (Taylor *et al.* 2006[a]) recruited 231 pregnant women with a history of allergic disease as indicated by a positive allergen (SPT) into a RDBPC study. The probiotic (3×10^9 CFU/day *Lactobacillus acidophilus*) or placebo (maltodextrin alone) was perinatally administered daily for the first 6 months of life independent of maternal feeding regimens. The authors assessed mononuclear cell samples from 118 infants recruited to the study, for TLR-mediated cytokine responses to TLR-agonists, e.g. peptidoglycan (PGN), for TLR2, and lipopolysaccharide (LPS) for TLR4 in mononuclear cell supernatant. The TLR-agonist induced response was utilized as a surrogate of innate immune function in response to probiotic supplementation. Interestingly no differences were observed in the probiotic or placebo groups when cytokine responses were examined following stimulation with PGN or LPS. Similarly, no differences were seen in the antigen-presenting capacity of these

infants. The mean fluorescence intensities of human leucocyte antigen-DR on monocytes, B cells and dendritic cells subsets were not affected, nor were the percentage of circulating DC subsets affected by a 6-month supplementation with *Lactobacillus acidophilus*. The authors concluded that probiotic supplementation with *Lactobacillus acidophilus* did not alter early innate immune responses in this population at high risk of developing allergic disease. In the same cohort, (Taylor *et al.* 2006[b]) studied whether probiotic supplementation could modify allergen specific immune responses. Interestingly mononuclear cells of children who received the probiotics showed significantly reduced production of IL-5 and TGF-β in response to SEB stimulation. There were no significant effects of probiotics on either (Th1) or (Th2) responses to allergens or other stimuli. The only other effects observed were for reduced TNF-α and IL-10 responsiveness to HDM allergens in children receiving probiotics.

(Tang *et al.* 2009) studied the effects of probiotic administration on infant intestinal microbiota composition in infants at high risk of developing allergic disease. By randomising 122 pregnant women to receive either *Lactobacillius rhamnosus* LGG or placebo, from 36 weeks gestation until delivery, the authors determined the infant and maternal fecal bifidobacterium composition by terminal restriction fragment length polymorphism at birth and during the first 90 days of life. Interestingly at 90 days, infants in the LGG group were more frequently colonized with *Bifidobacterium longum,* and harboured a bifidobacterium fecal composition closely resembling that typically observed in healthy breast-fed infants. However the authors did not clearly assess how the increase in *Bifidobacterium longum* was attributable to prenatal LGG supplementation.

(Martino *et al.* 2008) studied colonization patterns and mucosal IgA production at 6 months of age in relation to early probiotic exposures, systemic immune development and childhood allergic outcomes in a cohort (n = 189) receiving either *Lactobacillus acidophilus* or a placebo since birth. Interestingly, in this study children who developed sensitization to one or more allergens by 12 months of age had significantly higher total IgA levels at 6 months, irrespective of probiotic treatment, implying no connection between colonization patterns and total or secretory IgA levels on atopic sensitization, atopic dermatitis or food allergy.

Breastfeeding and the use of yoghurt or probiotic supplements were independently associated with significantly higher proportions of 'favourable' gut microbiota lactobacilli and bifidobacteria species, attenuated cytokine production and increased expression of regulatory cell cytokines such as TGF-β.

In a RDBPC study involving 253 Asian infants at high risk for developing allergic disease, (Soh *et al.* 2009) assessed the effect of probiotic supplementation during the first 6 months of life on eczema and allergic sensitization by age 1. Infants received at least 60 mL of commercially available cow's milk formula with or without (*Bifidobacterium longum* 1×10^7 CFU)/g and *Lactobacillus rhamnosus* 2×10^7 CFU/g) daily for the first 6 months. Clinical evaluation was performed at 1, 3, 6 and 12 months of age, with serum total IgE measurement and SPTs conducted at the 12-month visit. Un-informatively the incidence of eczema in the probiotic group at 22% was similar to that in the placebo group at 25% and the prevalence of allergen sensitization showed no difference. Total IgE levels were also similar in both groups. Atopic eczema (with sensitization) in the probiotic (7.3%) group was comparable to the placebo group (5.8 %.), indicating that post-natal probiotic supplementation had no effect on eczema or allergen sensitization in infants at risk of allergic disease.

In summary data in support of the application of perinatal supplementation of the neonate presenting with a familial history of atopic disorder to attenuate the risk of the atopic disorder are mixed. Some studies highlight a protective effect of probiotic supplementation on the development of atopic dermatitis, some imply that there is no significant effect of probiotic supplementation on atopic disorder and some supplemental studies even exacerbating the risk of atopic or respiratory disorder (Taylor *et al.* 2007) (See Table 1.6.1). To add further complexity to the rationale of probiotic supplementation for the protection against allergic disorder; probiotic effects in supplemental trials appear to be strain and consortium specific. It is currently not clear whether one specific probiotic strain or a group of strains are important in protecting against atopic disorder or whether probiotic supplementation will be dependent on the disease specific immune signature of a particular atopic disorder.

1.6.3 Perinatal supplementation with probiotics and prevention of allergic disease: laboratory measures

Different groups have complemented their clinical studies with attempts to understand the immunological mechanisms that underlie anticipated immunomodulatory effects of probiotics on measures of allergy. These include immunological analysis of breast milk, umbilical cord and infant blood. Studies of blood from infants participating in probiotic studies generally investigate levels of candidate mediators in plasma/serum such as IgE, the inflammatory marker C-reactive protein CRP, eosinophilic cationic proteins or cellular immune activity whole blood or mononuclear cell cultures at the cytokine level. For example the response of maternal and umbilical cord blood MNCs to IL-2, β-lactoglobulin (βLG) or inactivated LGG was examined in an LGG supplementation cohort (Kopp *et al.* 2008). Interestingly, LGG induced release of IL-10 and IFN-γ but not IL-13 by cord and maternal MNCs, with no significant differences between the study groups for any of the other responses measured.

In the study by (Niers *et al.* 2009), employing a consortium of *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus lactis,* whole blood culture responses to the T cell stimuli anti-CD3/CD28 or PHA were assessed at 3 months of age. Anti-CD3/CD28-stimulated IL-5 was significantly reduced in the probiotic group compared to the placebo group. This is noteworthy as IL-5 is involved in the differentiation of eosinophils and supports prolonged eosinophil survival. However the authors did not measure the extent of eosinophila in the study groups. There were no differences in IL-10 or with any PHA-stimulated responses.

Analysis of cord blood plasma from newborns in a New Zealand cohort revealed that neonates of mothers receiving probiotic had significantly enhanced cord blood IFN-γ and a significantly higher proportion had detectable cord blood IFN-γ levels, compared with the placebo group (Prescott *et al.* 2008). This pattern was evident for *Lactobacillus rhamnosus* and cord sCD14 was lower for *Bifidobacterium lactis.* Development of atopic eczema in the first 2 years of life was associated with elevated cord plasma IL-13 and IL-10 regardless of probiotic supplementation.

Perinatal probiotic supplementation has also been associated with increased circulating levels of the CRP, along with increases in total IgA, total IgE and IL-10 at 6 months of age but no differences in IL-2, IL-4, IL-6, TNF-α or IFN-γ (Marschan *et al.* 2008). Elevated CRP at 6 months was associated with decreased risk of eczema and allergic disease at 2 years of age.

A study by (Taylor *et al.* 2007) supplemented neonates post-natally with *Lactobacillus acidophilus,* and then examined early markers of allergic disorder. Interestingly, inhalant sensitization and childhood dermatitis at the 2.5 year follow up phase was associated with higher proportions of circulating $CD4^{+}CD25^{+}$ regulatory T-cell populations and higher allergen-induced FOXP3 levels at 6 months. However the authors did not attribute this observation to a cytokine mediated immunomodulatory effect of *Lactobacillus acidophilus* influencing regulatory T cell development, but rather to the chronic inflammatory immune landscape of inhalant sensitization and childhood dermatitis, associated with increased early expression of regulatory T-cell markers. Furthermore, children with dermatitis were reported to have lower PHA induced IFN-α, IL-10 and IL-6 responses. However, it was not apparent in this study, whether *Lactobacillus acidophilus* influenced PHA induced responses.

In studies utilising breast milk, analysis of either TGF-β1 or TGF-β2 often forms the focus. LGG supplementation pre-natally and post-natally was associated with significantly elevated TGF-β2 but not TGF-β1 levels in breast milk of probiotic but not placebo taking mothers. Interestingly, the presence of cord blood IgE correlated with elevated TGF-β2 in breast milk (Rautava *et al.* 2002). Elevated TGF-β2 was also found in colostrum from a group of women who had been taking a consortium of LGG and *Bifidobacterium lactis* from the first trimester. This difference did not persist at 1 month post-partum, and there were no differences in any of the other mediators measured- sCD14, IFN-γ, TNF-α, IL-10, IL-6, IL-4, and IL-2 (Huurre *et al.* 2008). In contrast lower TGF-β2 and elevated IL-10 were found in colostrum but not milk collected one month postpartum in a study of supplementation with *Lactobacillus reuteri* (Bottcher *et al.* 2008). The effect on TGF-β2 was most pronounced in mothers with *L. reuteri* recovered in their faeces. Total and secretory

IgA, TNF-α, and sCD14 were also measured, and no differences between probiotic and placebo groups were observed. Breast milk samples from the New Zealand cohort in which two different species of probiotic were compared (*Lactobacillus rhamnosus* HN001 or *Bifidobacterium lactis* HN019) were analysed for total IgA, IL-13, IFN-γ, IL-6, TNF-α, IL-10, TGF-β1 and sCD14 (Prescott *et al.* 2008). In this cohort TGF-β1 levels were higher in milk collected 1 week postpartum for both probiotic groups but this was significant for the *B. lactis* group only. Probiotic fed mothers were more likely to have total IgA and IL-6 detectable in their breast milk confirming the strain specific immunomodulatory effects of probiotic supplementation on the immunological composition of human breast milk.

These studies indicate that investigators can successfully demonstrate that supplementation with probiotics in pregnancy has the potential to influence fetal immune parameters as well as immunomodulatory factors in breast milk. Determining how this relates mechanistically to clinical effects of probiotics is now the challenge. Recent studies challenging the general view of a sterile intrauterine existence in which microbial colonisation of the gut begins after delivery might contribute to these effects (Satokari *et al.* 2009). For instance, DNA transcripts of *Bifidobacterium* and *Lactobacillus* have been detected in the human placenta, irrespective of mode of delivery: of 34 placentae studied bifidobacterial DNA was detected in 33 and *L. rhamnosus* DNA in 31 (Satokari *et al.* 2009). This was interpreted as horizontal transfer of bacterial DNA from mother to foetus via the placenta. This observation is significant because bacterial DNA contains unmethylated CpG oligodeoxynucleotide motifs capable of inducing immune responses. Exposure to bacterial DNA may programme the infant's immune development during foetal life, earlier than previously considered (Satokari *et al.* 2009). These observations also reinforce the notion of the existence of evolutionary conserved mechanisms favouring microbial development of a probiotic rich intestinal tract. Given the findings of (Satokari *et al.* 2009) it is interesting that the work of (Patni *et al.* 2009) demonstrated that whilst transcripts for TLR9, a receptor involved in the innate immune response to unmethylated DNA, were detectable in the term and preterm placenta, a functional cytokine output could not.

Table 1.6.1: Table of pre-natal, perinatal and post-natal probiotic studies

Author	Study	Age at enrollment, health status and length of study	Variables measured	Effects of probiotics
(Kalliomaki *et al.* 2001)	(RDBPC with N =159 participants); 2 arms: (1) Placebo: capsule of microcrystalized cellulose (2) Experimental: capsule of LGG 1x ^{10}CFU/ Capsule (2 capsules per day)	0-6 Months Family history of atopic disease 6 months (2 year follow-up)	1. Atopic eczema presence 2. Serum total IgE 3. Serum specific IgE 4. Skin prick test	1. LGG supplementation significantly reduced the frequency of atopic disease to half of that of the placebo-group
(Rautava *et al.* 2002)	(RDBPC); 2 arms: 1. Control/placebo: microcrystalline cellulose 2. Experimental probiotic LGG capsules 2×10^{10} CFU/d N = 62 mother infant pairs	Mother age (n/a) Infants 1 day of life Family history of atopy (4 weeks prior to birth until infant reached 3 months)	1. Serum total IgE measured from cord blood 2. TGF-β1 and TGF-β2 measured from breast milk at 3 months 3. Risk of atopic eczema	2. TGF-β2 higher in probiotics group 3. Risk significantly reduced in probiotics group compared to placebo
(Kalliomaki *et al.* 2003)	(RDBPC); 2 arms: 1. Control: capsule of microcrystalized cellulose 2. Experimental-capsule of LGG 1x 10^{10} CFU/capsule 2 capsules per day N = 159	0-6 months Family history of atopic disease 4 year follow-up	1. Atopic eczema presence 2. Bronchial inflammation via exhaled nitric oxide 4. Skin prick test	1. LGG supplementation significantly reduced the relative risk of atopic eczema to 0.57 frequency of atopic disease to half of that of the placebo-group
(Kalliomaki *et al.* 2007)	Supplementation protocol: as above 116/132 of those who completed at 2 years of age studied at 7 years of age		Assessing persistence of potential to prevent eczema at 7 years. 2. rhinitis; asthma; SPT	1.Cumulative risk for developing eczema in first 7 years significantly lower in probiotic group 3.More children in probiotic group had rhinitis

(Rinne *et al.* 2005)			Microbiology and laboratory data - faecal, infant blood and breast milk analysis	1. Fecal Bifidobacterium and Lactobacillus/Enterococcus counts significantly higher in breastfed than formula-fed infants at 6 months. 2. At 3 months, total number of IgG-secreting cells in breastfed infants supplemented with probiotics exceeded those in breastfed infants receiving placebo correlating with concentration of sCD14 in colostrum. Total numbers of IgM-, IgA-, and IgG-secreting cells at 12 months were significantly higher in infants breastfed exclusively for at least for 3 months and supplemented with probiotics as compared with breastfed infants receiving placebo.

		health status and length of study	**measured**	**probiotics**
(Huurre *et al.* 2008)	*Lactobacillus rhamnosus* strain GG and *Bifidobacterium lactis* Study group: N= 171 mother-baby pairs Supplementation protocol: 1 x 10^{10} CFU/day or placebo (microcrystalline cellulose and dextrose anhydrate)	Women with atopic disease First trimester to end of exclusive breast feeding.	Primary outcomes: Breast milk cytokines & atopic sensitisation of infants at 6 and 12 months of age Other outcomes: Breast milk immediately after birth and at 1 month from 140 (probiotic – 72; placebo – 68).	1. TGFβ2 elevated in colostrum of probiotic group but not significant and not at 1 month.
(Niers *et al.* 2009)	*Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus lactis* Study group Supplementation protocol: 3 x 10^9 CFU/day (1 x 10^9 of each organism)	≥ 1 family member with atopic disease Last 6 weeks of pregnancy and postnatally for 12 months to offspring.	1. Impact on allergic disease at 2 years of age, with questionnaire and visit at 3, 12 and 24 months. 2.Weekly diaries, SPT at 24 months, total IgE at 3, 12 and 24 months, faecal analysis, whole blood culture (with anti-CD2/CD28; PHA) at 3 months of age	Parentally reported eczema reduced in probiotic group ($p = 0.035$); in those who consulted GP Significantly lower anti-CD2/CD28 induced IL-5 at 72 hours.
(Kim *et al.* 2009)	*Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus acidophilus* Supplementation protocol: 1.6 x 10^9 CFU of each in maltodextran/corn oil once daily Exclusive breast feeding to 3 months of age and then breast/formula.	≥ 1 family member with atopic disease 8 weeks before the expected delivery until 3 months after delivery and to baby from 4 to 6 months of age.	1. Eczema outcomes at 12 months of age; assessed at 3, 6 and 12 months. 2. Total and specific IgE at 12 months of age.	At 3 months fewer in probiotic group had eczema but not significant ($p = 0.086$). At 6 months half as many in probiotic group had eczema ($p = 0.053$). By 12 months probiotic < placebo, $p = 0.048$. Total IgE level and frequency of sensitisation comparable between the groups. Prevalence of atopic eczema halved in probiotics but $p = 0.223$.

(Wicken *et al.* 2008)	*Lactobacillus rhamnosis* or *Bifidobacterium lactis* Supplementation protocol: 6 x 10^9CFU/day of *L. rhamnosus* or 9 x 10^9CFU/day of *B. lactis* to mother from 35 weeks of pregnancy to 6 months after delivery if breast feeding. Baby received same from around birth to 2 years of age	≥ 1 family member with atopic disease	1. Cumulative prevalence of eczema and point prevalence of atopy (by SPT) at 2 years of age. 2.Faecal analysis	Those receiving *L. rhamnosus* had significantly reduced risk of eczema by 2 years of age and reduction in risk of high SCORAD note that those taking *L. rhamnosus* had significantly reduced IgE-associated eczema ($p = 0.04$).
(Prescott *et al.* 2008)	Organisms: as above Study group: as above Supplementation protocol: as above Primary outcomes		Cord blood and breast milk cytokines (3 – 7 days, 3 months and 6 months postpartum) from n = 30 – 40 in groups from above clinical study. Measured IL-13, IFN-γ, IL-6, TNF-α, IL-10, TGF-β1 and sCD14 in cord blood and breast milk as well as total IgA in breast milk.	If mother received probiotic cord blood IFN-γ increased and more subjects had detectable cord blood – same pattern for both organisms but significant for *L. rhamnosus* Cord sCD14 lower in *B. lactis* group. TGF-β1 higher in 1 week milk samples in both probiotic groups but significant for *B. lactis*. Probiotic fed mothers more likely to have total IgA and IL-6 detectable in their breast milk.

Author	Study	Age at enrollment, health status and length of study	Variables measured	Effects of probiotics
(Abrahamsson *et al.* 2007)	Organisms: *Lactobacillus reuteri* Supplementation protocol: 1 x 10^8 CFU/daily or placebo from 36 weeks of pregnancy until delivery and then same to baby to 12 months of age.	Study group: ≥ 1 family member with atopic disease	Primary outcomes: Allergic disease to 2 years of age with SPT/specific IgE to food.	Probiotic group has less IgE-associated eczema in 2nd year (8% vs. 20%, p = 0 .02) – only significant at 2 years of age but consistently lower prevalence in probiotic group. Egg white specific IgE less often detectable in probiotic group only significant at two years of age. Infants in probiotic group less likely to be SPT+, this was significant in those with allergic mothers. Cumulative incidence of wheeze similar at 2 years
(Bottcher, *et al.* 2008)	Organisms: as above Study group: as above Supplementation protocol: as above		Colostrum (day 3) and mature milk (1 month) from above – total and secretory IgA, TGFβ1, TGFβ2, IL-10, TNF, sCD14 and Na/K ratios	n = 109; 54 probiotic, 55 placebo. Probiotic group had significantly lower TGFβ2 and significantly higher IL-10 in colostrum (but not mature milk). Infants receiving breast milk with lower TGFβ2 less likely to become sensitised during first 2 years.

(Kopp, *et al.* 2008)	Organism(s): *Lactobacillus rhamnosus* strain GG Supplementation protocol: 1 x 10^{10} CFU/day to mother from 4 – 6 weeks before delivery and after birth to breast feeding mothers for 3 months and then to neonates for another 3 months; otherwise to infant for 6 months.	Study group: ≥ 1 family member with atopic disease	Atopic dermatitis at 2 years of age Other outcomes: Severity of AD, wheezing bronchitis and allergic sensitisation.	Children with recurrent episodes of wheezing bronchitis more frequently in probiotic group No effect on eczema but increased recurrent wheezing bronchitis.
(Kopp *et al.* 2008)	Organism(s): as above Study group: as above Supplementation protocol: as above Other outcomes:		Performed *in vitro* studies using cord blood and maternal peripheral blood MNCs from those taking probiotic or placebo and exposed to IL-2, β-lactoglobulin (βLG) or inactivated (70% ethanol) LGG. Cytokine responses by mononuclear cells (MNCs) – *in vitro and in vivo*	LGG induced release of IL-10 and IFN-γ but not IL-13 by cord and maternal MNCs
(Kukkonen, *et al.* 2007) Helsinki and Tampere,	Organism(s): *L. rhamnosus GG* (5 x 10^9 CFU); *L. rhamnosus* LC705 (5 x 10^9 CFU); *B. breve* (2 x 10^8 CFU); *Propionibacterium freudenreichii* (2 x 10^9 CFU) Supplementation protocol: Mother took 2 capsules/day from 2 – 4 weeks before delivery and then baby 1 capsule/day from birth to 6 months of age – babies also given galacto-oligosaccharides daily	Study group: ≥ 1 family member with atopic disease	Cumulative incidence of any allergic disease (food allergy, eczema, asthma and allergic rhinitis) and IgE-associated disease over first 2 years Eczema and IgE sensitisation (SPT at 6 and 24 months, specific IgE at 24 months); faeces at meconium, 3, 6 and 24 months	Eczema and atopic eczema less likely in probiotic group. After adjustment for confounders any IgE-associated disease, eczema and atopic eczema significantly lower in probiotic group.

1.7 Need for the study

A vast body of evidence has accumulated linking consumption of probiotic rich fermented foods, with enhanced immune tolerance mechanisms. Probiotics are therefore postulated to have a therapeutic role in pathologies with a dysregulated immune component. Despite unprecedented research efforts to determine the impact of probiotic organisms in health and disease; commonly accepted paradigms for their immunomodulatory mechanisms are unclear. To date, research into the role of probiotic bacteria in allergic disorder, is based primarily upon *in vitro* data suggesting an intrinsic capacity of probiotics to attenuate experimentally induced Th2 immune responses by enhancing Th1 inflammatory and regulatory mechanisms (Cross *et al.* 2001) .

However, due to the vast diversity and quantity of probiotic micro-organisms existing within the GIT, attempts to determine the immunomodulatory capacity of a probiotic optimised diet *in vivo* remain inconsistent and highly controversial with results ranging from beneficial to inconclusive health benefits. Additionally, the premise that many potentially probiotic micro-organisms are currently un-culturable using available techniques adds further complexity to the field. Observations that probiotics exhibit strain specific effects, have inspired researchers to ascertain favourable cocktails of probiotics necessary to mediate beneficial immunomodulatory effects in immune dysregulated disease settings such as IgE mediated atopic disease and gastrointestinal disorders where probiotic imbalances are implicated (Cross *et al.* 2001).

Although there is a sound theoretical basis for anticipating benefits of probiotic supplementation; existing data are insufficient to support recommendation of probiotics as a part of standard therapy for allergic conditions. Furthermore, although several studies appear to show a benefit in prevention of atopic eczema this literature review has indicated that some studies refute these observations, showing no clear preventive effect on sensitization, nor any allergic disease other than eczema.

The term ''probiotic'' has therefore been loosely applied to include bacterial strains with little documented immunomodulatory capacity or controlled studies to support health benefit claims. Moreover it is not known whether effects in experimental systems have any clinical relevance. Explanations for the varied results between studies include host factors (including genetic differences in microbial responses and allergic predisposition) and other environmental factors, such as general microbial burden, individual microbiota, diet (including consumption of prebiotic substances), and treatment with antibiotics. As more studies are completed, these factors are likely to make robust meta analyses problematic to perform (Prescott and Bjorksten 2007).

Chapter 2: General Materials & methods

Methodologies employed in this thesis can be divided into two broad strategies with a common goal: to characterise the immunomodulatory effects of a study consortium of probiotic bacteria (*Lactobacillus salivarius*, *Lactobacillus paracasei*, *Bifidobacteria bifidum* and *Bifidobacteria infantis*) to model how indigenous gastrointestinal tract bacteria might benefit immunity. The *in vitro* arm of this study employed a cross-sectional population of healthy neonates, healthy pregnant and non-pregnant women, and healthy men (section 2.1). The *in vivo* arm focused on discerning the immunomodulatory effects of probiotic supplementation using a cohort of newborns (n = 438 section 2.2).

2.1 Materials & methods for *in vitro* analysis

2.1.1 Optimising culture conditions for use of probiotic bacteria in mammalian cell culture systems.

In the laboratories of the industrial partner (Cultech Ltd), an assessment of how well strains of the study consortium tolerated conditions typically used for culture of mammalian cells was first determined. The experimental co-culture conditions for assessing the immunomodulatory effects of the study consortium, upon interaction with mononuclear cells, were then analysed in *vitro.*

The consortium consisted of the bacterial micro-organisms:

- ***Lactobacillus salivarius*** CUL61 (national collection of industrial, food and marine bacteria (NCIMB) 302111);
- ***Lactobacillus paracasei*** CUL08 (NCIMB 30154);
- ***Bifidobacteria animalis*** subsp. lactis CUL34 (NCIMB 30172); and
- ***Bifidobacteria bifidum*** CUL20 (NCIMB 30153).

2.1.2 Assessing the viability of the study consortium in mammalian cell culture systems.

The ability of bacterial organisms of the study consortium to survive and proliferate in standard cell culture media commonly used to culture human mononuclear cells was assessed. Survival of probiotic strains in both the absence and presence of mononuclear cells was quantified by total viable counts (TVC) on agar plates. 1×10^{10} CFUs of the study consortium was added to culture medium and then serially diluted down to 1×10^{7} CFU in 4.5 ml of culture medium. The culture media used were: RPMI1640 with Glutamax and phenol red pH indicator, and AIM/V (both Invitrogen, UK). At time 0 (T_0), and after 6 (T_6), 24 (T_{24}) and 48 hours (T_{48}), 500 µl from the 1×10^{7} CFU dilution was serially diluted in maximum recovery diluent (MRD, Oxoid, UK) to concentrations ranging from 1×10^{6} to 1×10^{2} and used for the TVC (Figure 2.1).

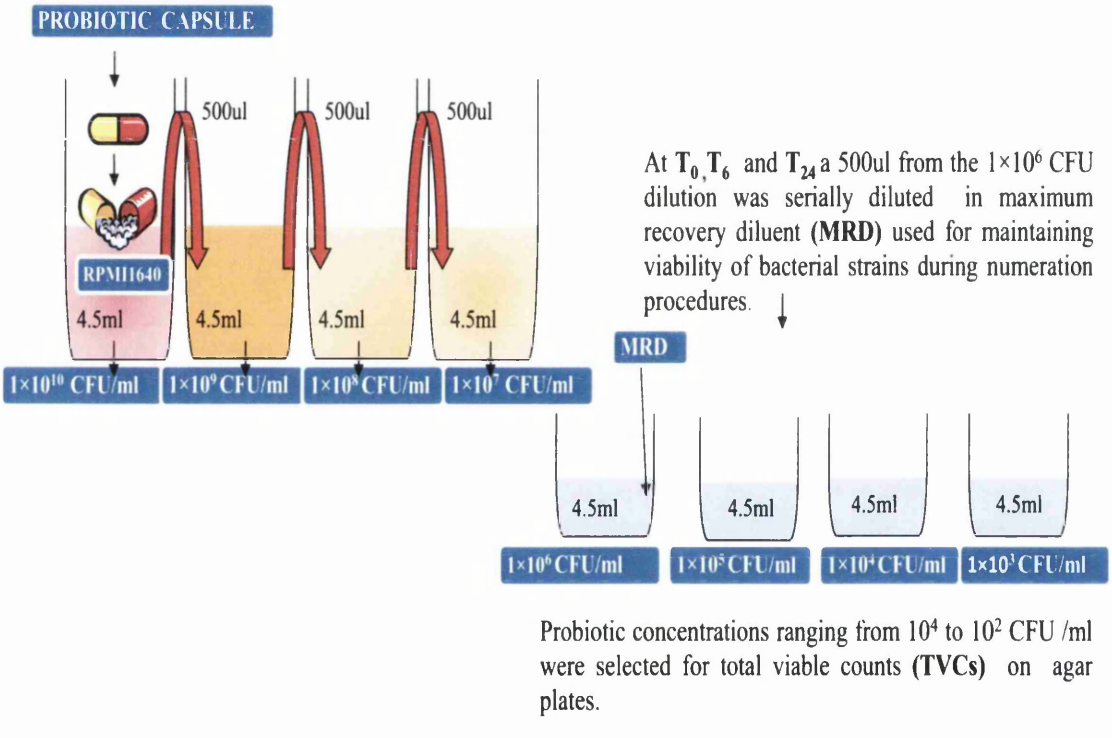


Figure 2.1: Dilution procedure for 1×10^{10} CFUs of the study consortium for enumeration purposes.

Total viable counts (TVCs) were determined by adding selected dilutions of the study consortium onto MRS or MRSx agar plates (provided by the industrial partner) using a modified Miles and Misra (Miles *et al.* 1938) plate count technique. Agar plates where then transferred to a 37^{0}C anaerobic incubator for 2-3 days to allow viable organisms to grow. Members of the genera Lactobacillus and Bifidobacteria can be selected on solid culture media that have an acidic pH such as MRS under anaerobic conditions (Tannock 1999). MRSX agar plates select for bifidobacteria species whereas MRS agar plates support abundant growth of *Lactobacillus* species exclusively.

To determine the total number of viable colonies present at each time point the number of bifidobacteria and lactobacillus species were summed. The following formula was used to obtain the TVC:

$$TVC = (\text{total number of colonies} \times 10) \times 10 \times \text{dilution factor}$$

2.1.3 Viability of study consortium in mammalian cell culture medium during co-culture with mononuclear cells

Adult peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation as described below. PBMCs (5×10^5) were co-cultured with concentrations of the study consortium ranging from 1×10^7 to 1×10^4 CFU/ml. Doses were prepared by dilution of 500mg of a 1×10^{10} CFUs freeze-dried active product in RPMI1640 with Glutamax and phenol red pH indicator supplemented with 5% fetal bovine serum (FBS; Invitrogen) and 0.5mM 2-mercaptoethanol (Invitrogen)(Figure 2.2).

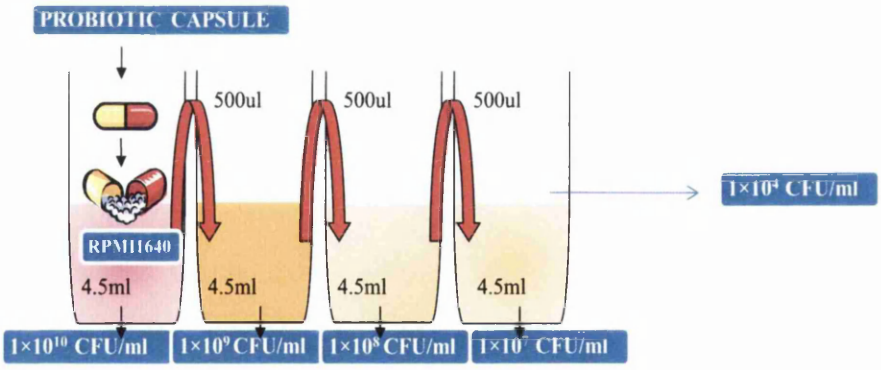
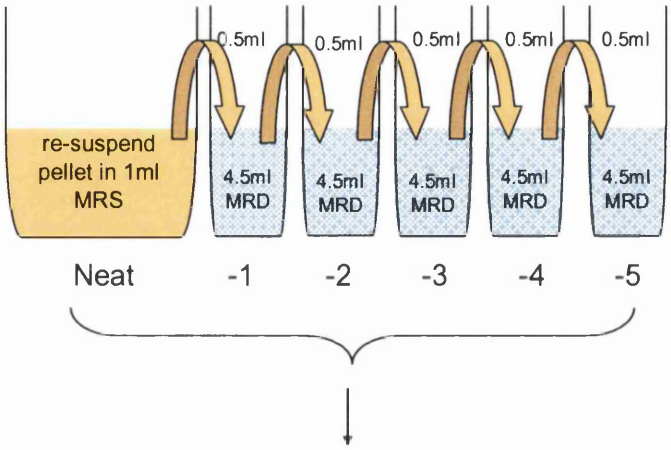


Figure 2.2: Illustration of the dilution process of the study consortium in RPMI1640

At each time point (T_0, T_6, T_{24}, and T_{48}) the co-culture was transferred to a microfuge tube and centrifuged for 7 minutes at 3200 x *g* to obtain a pellet containing a mixture of PBMCs and the study consortium. The pellet was re-suspended in 1 ml MRS agar broth (a liquid phase growth media for lactobacillus

and bifidobacteria species) and then serially diluted in MRD for total viable counts on each sample dilution as described in 2.1.2.1 (Figure 2.3).



Maximum recovery diluent **(MRD)** is an isotonic diluent containing a low level of peptone used for maintaining the viability of bacterial organisms for at least 1hr during dilution procedures

Five 10µl drops of each dilution were added unto MRS and MRSx agar plates and left to incubate for 3days in an anaerobic incubator. The number of CFUs present at each time point during the co-culture with PBMCs was calculated as previously described in this section.

Figure 2.3: Illustration of the dilution process of the PBMCs-consortium pellet in maximum recovery diluent.

2.1.4 Flow cytometric analysis of the interaction between probiotic microorganisms and mononuclear cells

This part of the study investigated the application of flow cytometry for determining the viability of the study consortium, especially after the effects of co-culture with mammalian cells. The study consortium was labelled with carboxyfluorescein diacetate succinimidyl ester (CSFE). CFSE is a colourless non-fluorescent compound which passively diffuses into cells. Intracellular esterases encountered during diffusion cleave acetate groups present within the CFSE molecule yielding a highly fluorescent carboxyfluorescein succinimidyl ester which reacts with intracellular amines forming florescent conjugates well retained in viable cells.

Excess un-conjugated reagent and by-products passively diffuse back into the culture medium.

CellTrace™ CFSE cell proliferation kit (Invitrogen) was used to label the consortium bacteria. Briefly, CFSE was prepared according to the manufacturer's guidelines: the recommended working concentration was 50 μM and was prepared by using 18 μl of dimethyl sulphoxide (DMSO; Sigma) to dissolve the contents of a vial containing CellTrace™ CFSE to create a 5 mM stock solution. This underwent a further 1 in 50 dilution (12 μl of the 5mM stock in 588 μl sterile PBS) to yield a 0.1 mM solution of which 100 μl was added to a 100 μl preparation of the study consortium containing 1×10^8 CFUs/ml (final CFSE concentration of 50 μM). The mixture was left to incubate in ice for 10 minutes; cold RPMI1640 was added prior to a further 5 minute incubation and then centrifugation at 4000 x g for 10 minutes. This washing step was repeated twice. Finally the pellet containing 1 x 10^7 CFUs of CFSE labelled bacteria was re-suspended in 100 μl of RPMI1640/Glutamax supplemented with 5% FBS and 0.5Mm 2- Mercaptoethanol (2-ME) and co-cultured with 0.5 x 10^5 mononuclear cells (MNCs; prepared as described in section 2.1.3) in a total of 500μl/well of a 48 well plate.

At T_0 and T_{24} the contents of the co-culture was transferred to a microfuge tube and spun at 510 x *g* for 7 minutes at 4°C, supernatants were removed and the pellet was re-suspended in 50 μl of FACS buffer. Where necessary, relevant antibodies were added to each tube and left to incubate on ice for 30 minutes. The antibodies used were: anti-CD45: APC, anti-CD14: PE-Cy5.5 or anti-CD3: PE-Cy5.5 (Caltag; Invitrogen). After incubation, 3 ml of FACS buffer was added to each tube and tubes were spun at 510 x *g* for 7 minutes at 4°C. The supernatant was poured away with care taken to not disturb the cell/bacteria pellet which was re-suspended in 200 μl of FACSFix (BD CellFIX diluted 1 in 10 with distilled water; BD Biosciences). Cells/bacteria were acquired and analysed within 24 hr of fixing using a FACSAria flow cytometer and CellDiva software (version 5.0.3; both BD Biosciences).

2.1.5 Density gradient centrifugation of umbilical cord or adult peripheral blood for the isolation of mononuclear cells

Mononuclear cells of the haematopoietic system are a critical component of the immune system involved in both innate and adaptive immune responses. Mononuclear cells are distinguished from other leukocytes in that they do not contain granules and hence are called collectively agranulocytes, encompassing predominantly lymphocytes and monocytes. Mononuclear cells used in this thesis were extracted from whole blood using ficoll, a hydrophilic polysaccharide that separates layers of blood yielding mononuclear cells depleted of red blood cells and most granulocytes located under a layer of plasma (Delves *et al.* 2006) (Figure 2.4). The resulting cell population is termed adult peripheral blood mononuclear cells (PBMCs) if the blood sample is from an adult donor or cord blood mononuclear cells (CBMCs) if from the umbilical cord.

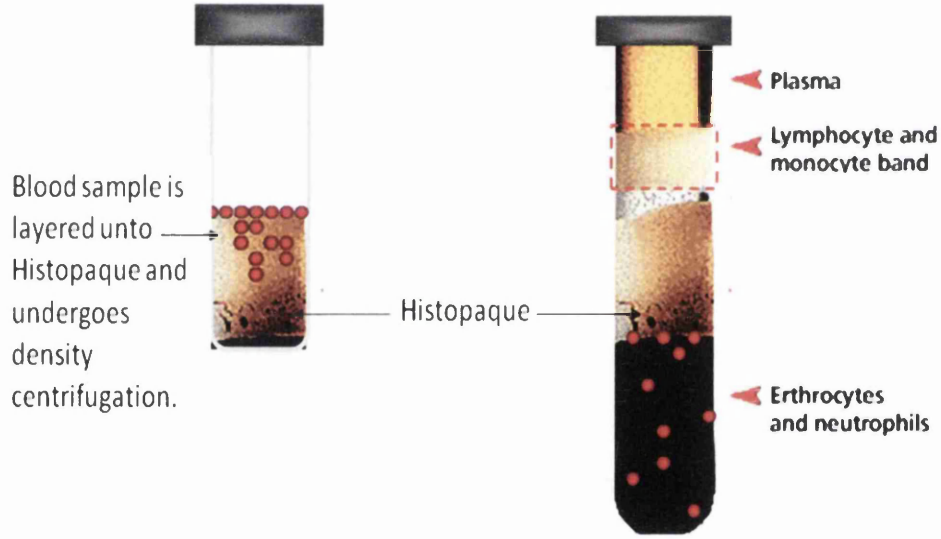


Figure 2.4: Illustration of the separation of mononuclear cells, plasma and erythrocytes and granulocytes by density gradient centrifugation method.

All handling of umbilical cord blood and adult peripheral blood took place in a Class II tissue culture cabinet to limit contamination. Undiluted heparinised blood was layered onto an equal volume of Histopaque®-1077 (Sigma, USA) in a 50 ml Falcon tube (Greiner Bio-one, Germany) and centrifuged at 805 x *g* for 20 minutes at room temperature (brake off). The plasma was then removed, filtered (0.2 µm

polyethersulfone filter; Millipore, MA, USA), aliquoted into well-labelled tubes and stored at -20°C until later analysis. The layer of cells at the interface was removed into a 30 ml Universal (Greiner Bio-one, Germany), the tube topped up with RPMI 1640/Glutamax (Invitrogen, UK) and centrifuged at 515 x *g* for 10 minutes at room temperature. Following centrifugation the supernatant was discarded and the cell pellet resuspended in RPMI 1640/Glutamax, prior to another centrifugation at 515 x *g* for 7 minutes at 4°C. The supernatant was discarded and cells were gently resuspended in an appropriate volume of RPMI 1640/Glutamax supplemented with 0.5 mM 2-mercaptoethanol and 5% FBS (both Invitrogen, UK) before being manually counted on a haemocytometer. This approach is summarised in Figure 2.5.

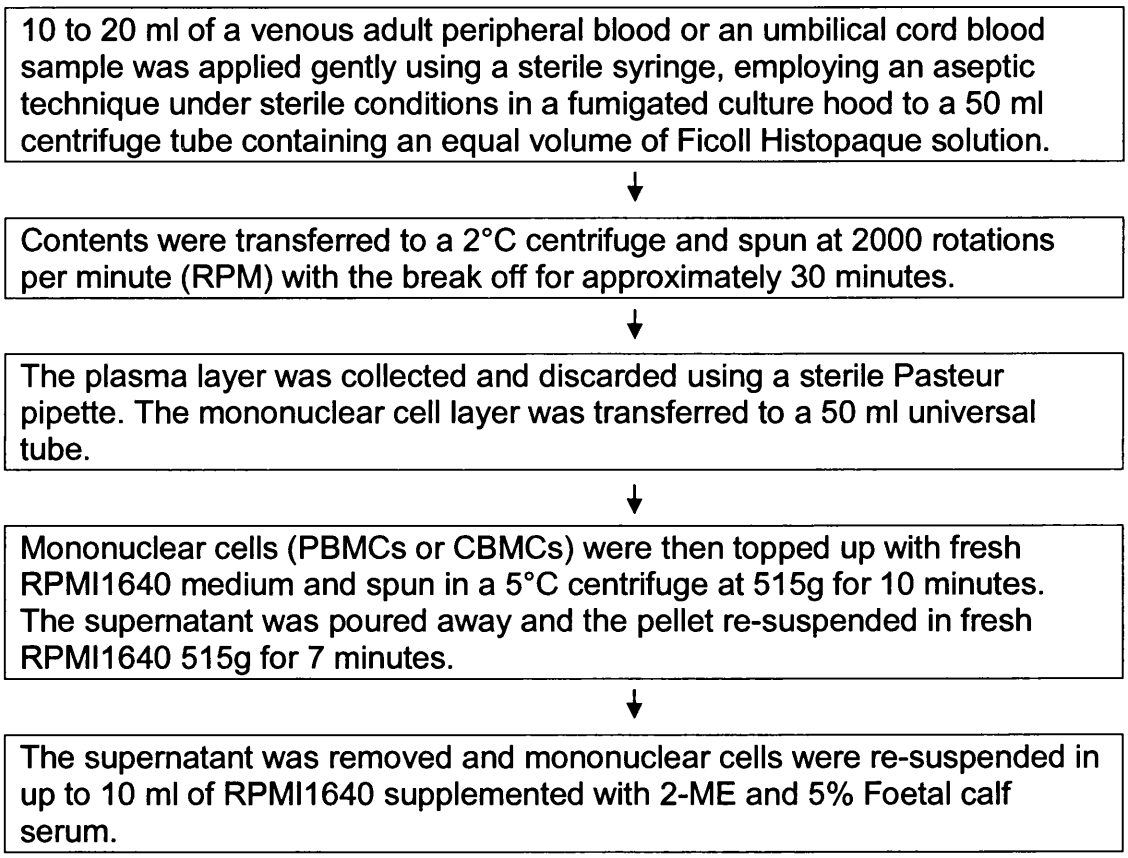
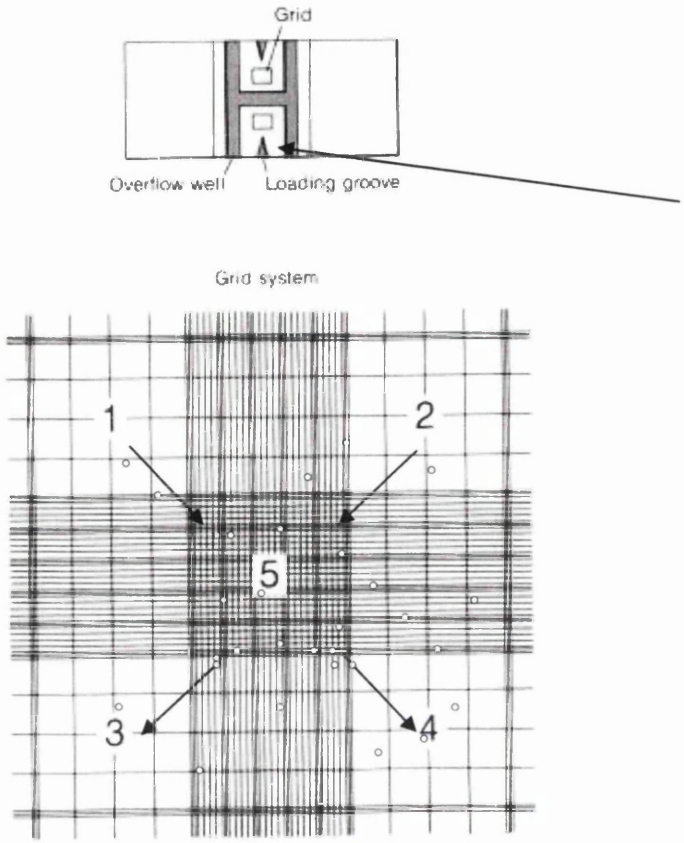


Figure 2.5: Flow chart for isolation of mononuclear cells from heparinised whole blood using density gradients centrifugation.

Following re-suspension, a 10 µl sample of mononuclear cells was transferred to a C-Chip haemocytometer (Digital-Bio, Korea) for enumeration. Note that a cell viability dye was not included as extensive experience in the research group has found that with freshly prepared mononuclear cells the number of non-viable cells is

negligible. Five squares were counted (See Figure 2.6) and then cell numbers were calculated: (number of mononuclear cells counted × 5) × 10^4 per ml. Based on this count MNCs were diluted in accordance with experimental requirements: typically, MNCs were used at 1×10^6/ml of cell culture media unless otherwise stated.



° = **Mononuclear cells**

Figure 2.6: Diagrammatic view of haemocytometer adapted from http://www.pathology.washington.edu/research/labs/rabinovitch/flowroom/protocols/RabLabManual_files/image002.jpg

2.1.6 *In vitro* dose response studies.

Mononuclear cells were co-cultured with varying doses of the study consortium to model haematopoietic cell and probiotic interactions in terms of the spectrum of cytokines released from mononuclear cells upon exposure to potentially probiotic microorganisms. Purified mononuclear cells (MNCs) at 1×10^6/ml in RPMI1640/Glutamax supplemented with 5% FBS and 0.5 mM 2-mercaptoethanol

(referred to as 'complete medium' [CM] from here on; all Invitrogen as before) were prepared from umbilical cord and adult peripheral blood by density gradient centrifugation and co-cultured in duplicate in a 48 well plate (Greiner Bio-one) in the presence of varying of doses of the study consortium. MNCs were co-cultured in the presence of 10^6, 10^5, 10^4, 10^3 or 10^2 CFU/ml of the consortium of potential probiotic microorganisms prepared from the study capsules as described in 2.1.2. The 48 well plates (500µl/well) were then transferred to an incubator at 37°C in 5% CO_2-in-air for culture. After the first 6hr, 250 µl of media was removed and replaced with an equal volume of fresh CM. After further 18 hr incubation at 37°C in 5% CO_2-in-air, cell free culture supernatants were harvested: the contents of the wells were transferred to microfuge tubes which were centrifuged at 515g for 7 minutes to yield cell/bacteria free supernatants that were stored at -20°C until analysis. The cytokines measured using specific ELISAs (See section 2.1.12 and figure 2.9) were: TNF-α, IL-10, IL-6, IL-12p70, IL-1β, and TGF-β1.

For more detailed investigation of the mechanism of the responses observed in the above investigation the following approaches were taken:

2.1.7 Transwell studies

The requirement for contact between the study consortium bacteria and MNCs was assessed using transwells to separate the consortium and MNCs into distinct compartments during co-culture (Figure 2.7). Since the base of the transwell chosen is perforated with pores measuring 0.4µM in diameter and lactobacilli and bifidobacteria strains are approximately 1-2 µM in diameter contact between mononuclear cell and the consortium was fully restricted. CBMCs or PBMCs at 1×10^6/ml in complete medium were added to the wells of a 24 well plate (Greiner Bio-one). A transwell with 0.4 µM pores in the membrane (ThinCerts™ – tissue culture inserts, transparent; Greiner Bio-one) was placed into the well and the study consortium at 10^6 CFU/ml placed into the transwell. The plates were cultured at 37°C in 5% CO_2-in-air for 24 hours. Cell free supernatants were harvested as before from the MNC side of the transwell.

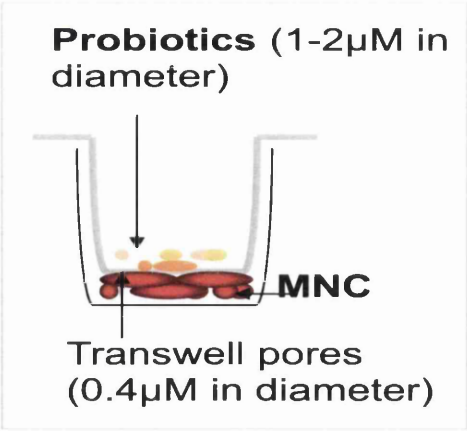


Figure 2.7: Illustration of the separation of mononuclear cells and potential probiotic bacteria of the study consortium using a transwell.

2.1.8 The interaction of $CD14^+$ monocytes with study consortium

Monocytes play crucial roles during immune defence, inflammation and tissue remodelling via their ability to phagocytise, process and present antigen, and by cytokine production. Therefore to explore the contribution of monocytes to the cytokine response to the study consortium, the ability of monocyte-depleted MNCs versus purified monocytes was compared. $CD14^+$ monocytes and $CD14^-$ depleted MNCs were prepared using magnetic microbeads and an autoMACS (both Miltenyi Biotec, Germany).

2.1.8.1 Principles of AutoMACS technology

MACS® technology is based on MACS magnetic microbeads, automated or manual MACS separators, and a MACS column matrix. When placed within a MACS separator, the MACS column matrix provides a magnetic field strong enough to retain specific magnetically labelled cell types within a cell sample.

MACS microbeads are small biodegradable superparamagnetic particles coupled to highly specific antibodies antibodies against proteins such as CD (cluster of differentiation) proteins. They have no known reported effects on the structure, function, or activity status of various cells types including mononuclear cells and therefore do not impact negatively on the overall experimental procedure. Cells can be prepared by positive selection – the target cell is labelled with magnetic

microbeads – or negative selection – the unwanted cells are labelled with magnetic microbeads to yield the target population that is not labelled. Once the total cell population of interest (e.g. MNCs) is labelled with magnetic microbeads, the cell suspension is place on a MACS column in a MACS separator. Cells labelled with magnetic microbeads will be retained in the column, while unlabelled cells will pass through the column to be collected as the unlabelled fraction. Retained/labelled cells can then be eluted from the MACS column after removal from the magnetic MACS separator.

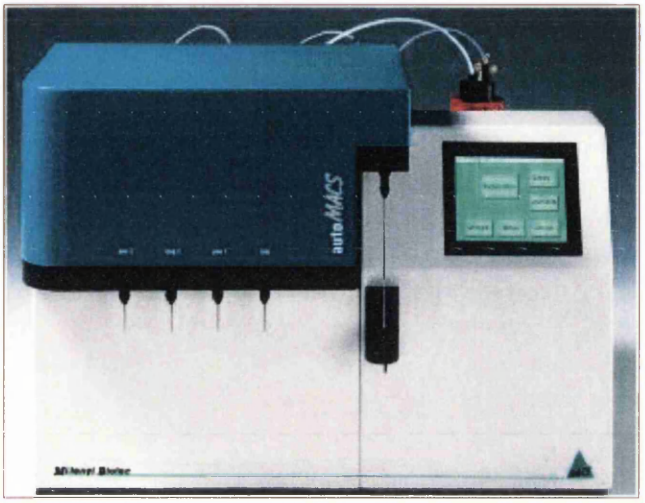


Figure 2.8: Picture of an AutoMACS cell separation machine as used in this study – (source of picture home.ncifcrf.gov/ccr/flowcore/automacs.jpg).

2.1.8.2 Isolation of $CD14^+$ monocytes and $CD14^-$ depleted MNCs

MNCs were first isolated from adult peripheral blood or umbilical cord blood samples by density gradient centrifugation over Histopaque®-1077 as described above (2.1.3). Contaminating red blood cells (RBCs) were then removed by magnetically separating Glycophorin (a sialoglycoprotein marker for RBCs) positive RBCs from the CBMC buffy coat. Due to the lighter density of RBCs in CBMC preparations compared to PBMCs preparations, this step was particularly critical for umbilical cord blood samples since the CBMC buffy coat preparation is always contaminated with RBCs. MNCs were labelled with glycophorin magnetic microbeads (Miltenyi Biotec) in accordance with the manufacturer's guidelines with one exception: 30 µl microbeads/10^7 CBMCs and 10 µl microbeads/10^7 PBMCs to allow for different levels of RBC contamination in the samples. Labelled MNCs

were then applied to the autoMACS and programme 'depleteS' used to deplete the MNC preparations of RBCs. RBC-depleted MNCs eluted from the magnetic column were then used for preparation of $CD14^+$ monocytes and $CD14^-$ depleted MNCs.

MNCs were counted and then labelled with CD14 magnetic microbeads in accordance with the manufacturer's guidelines (Miltenyi Biotec). Labelled MNCs were then applied to the autoMACS and collected as positively selected $CD14^+$ monocytes as one fraction, and negatively selected $CD14^-$ depeleted MNCs as the other fraction. To ensure complete depletion of $CD14^+$ monocytes from the $CD14^-$ depelted fraction, the cells in this fraction were pelleted by centrifugation, resuspended in MACS buffer (Miltenyi Biotec) and re-applied to the autoMACS. This time the programme 'depleteS' was used and only the negatively selected fraction was retained for further analysis. Cells in the two fractions of interest – $CD14^+$ monocytes and CD14-depleted MNCs - were then used for further analysis.

2.1.8.3 Co-culture of $CD14^+$ monocytes and $CD14^-$ depleted MNCs with study consortium

$CD14^+$ monocytes and $CD14^-$ depleted CBMC or PBMCs were counted using a haemocytometer and resuspended in completed medium at 1 x 10^6cells/ml. These cell fractions were then co-cultured in duplicate with study consortium bacteria at 10^6CFU/ml in a total volume of 500µl/well of a 48 well plate as before. After 24hr, cell free supernatants were harvested and stored at -20°C until analysis of TNF-α.

2.1.8.4 Flow cytometric evaluation of cells prepared using magnetic microbeads.

The AutoMACS protocol is compatible with downstream applications such as flow cytometry which was used to assess the purity of each fraction obtained via the purification procedure. In initial experiments, depletion of RBCs was determined using flow cytometry with anti-CD235a: PE (Caltag, Invitrogen). The purity of the $CD14^+$ monocyte fraction and the success of depletion of these cells from the $CD14^-$ depleted MNC fraction was monitored in every experiment using anti-CD14: FITC (Caltag). After counting, 5 x 10^5 cells were transferred to a FACS tube and FACS buffer added to give a total volume of 100 µl. Antibodies were added at pre-optimised titrations and the samples incubated on ice for 30 minutes in the dark.

After the addition of 3ml FACS buffer, cells were pelleted by centrifugation (510 x g, 7 minutes, 4°C) and then resuspended in 200 µl FACSFIX. Cells were acquired and analysed on a FACSAria flow cytometer using CellDiva software within 24hr as before.

2.1.9 Toll-like receptor (TLR) blocking experiments

The effect of blocking either TLR2 or TLR4 on the response by MNCs to the study consortium was determined. Functional grade antibodies (no azide, low endotoxin) known to neutralise the receptors of interest were used. MNCs (10^6/ml in complete medium) in a total volume of 500µl/well of a 48 well plate were pre-incubated with anti-TLR2 (clone TL2.1, mIgG2a), anti-TLR4 (clone HTA125, mIgG2a) or mouse IgG2a isotype control antibodies (all at final concentration of 20µg/ml; all eBioscience, San Diego, CA, USA). After 1hr, 10^6 or 10^5 CFU/ml of the study consortium was added at the outset of the culture. As a control MNCs exposed to a TLR4 ligand - lipopolysaccharide (LPS; 10ng/ml; Invivogen) - or a TLR2 ligand - peptidoglycan (PGN; 3µg/ml; Invivogen) – were used to control for success of TLR blocking. Cells/bacteria/antibodies were incubated for 24hr at 37°C in 5% CO_2-in-air prior to harvesting of cell free supernatants which were stored at -20°C until analysis using cytokine specific ELISAs.

2.1.10 Effects of the study consortium on stimulated mononuclear cell cytokine responses

The effects of the study consortium on cytokine production from MNCs following additional stimulation with the mitogen phytohaemagglutinin (PHA) or the superantigen staphylococcal enterotoxin B (SEB) was determined. This approach was chosen to model the effects of the study consortium on the Th1/Th2/Treg cytokine balance. PHA is a plant lectin that can be used in immunological assays to trigger proliferation and cytokine production by T lymphocytes. SEB is an exoprotein belonging to a family of structurally related proteins known as bacterial superantigens. These are a distinct class of antigens capable of stimulating a primary T cell response similar in magnitude to that in response to allogeneic MHC molecules. Superantigens can bind to both MHC and T cell receptor molecules and thereby stimulate proliferation and cytokine production by very large numbers of T

cells. Superantigens are unique to other protein antigens in that they are recognised by T cells without being processed into peptides that are captured by MHC molecules (Janeway 2003).

PBMCs or CBMCs were initially stimulated in the presence or absence of 10^6 CFUs of the study consortium for 24 hr as described above. PHA (1μg/ml, Sigma) or SEB (200ng/ml, Sigma) was then added to appropriate wells and the cultures left for a further 24 hr at 37°C in 5% CO_2-in-air before harvesting of cell/bacteria free culture supernatants. Supernatants were stored at -20°C until cytokine analysis using specific ELISAs. The cytokines selected for analysis were IFN-γ, IL-13, IL-4 and IL-17.

For more detailed investigation of the mechanism of the responses observed in the above investigation the following approaches were taken:

2.1.11 Studies using MNCs from pregnant women

To establish if pregnant women might differ to non-pregnant women in their response to the study consortium, MNCs were prepared from healthy term (> 37 weeks of gestation) pregnant women and non-pregnant women as described in 2.1.3. Dose response experiments as per 2.1.4 and the effect of pre-exposure to the study consortium on the response to PHA and SEB as per 2.1.9 were conducted.

2.1.12 Enzyme linked immunosorbent assay (ELISA)

2.1.12.1 Principles of ELISA

The enzyme linked immunosorbent assay (ELISA) is a biochemical technique used to detect the presence of an antibody or an antigen in a sample. An unknown amount of antigen binds to a surface or an optimised amount of capture antibody affixed to a surface, and then a second specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substrate is added so that the enzyme can convert to some detectable signal which is then measured, usually, as the optical density (Lequin 2005).

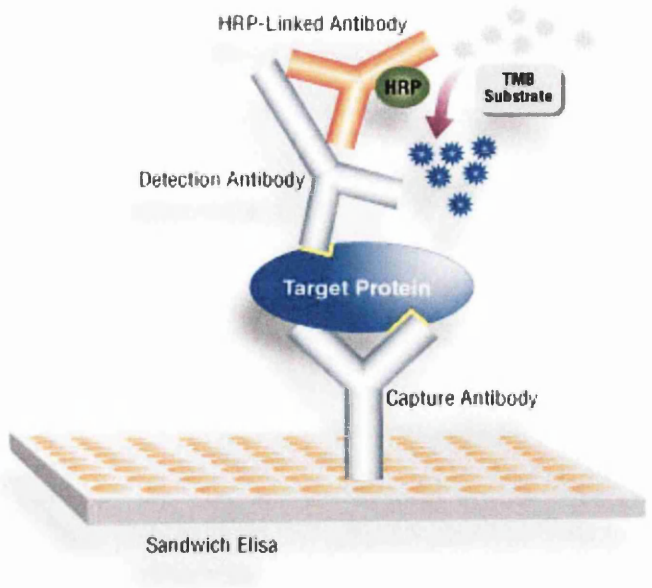


Figure 2.9: Diagrammatic representation of a sandwich ELISA.

An ELISA plate is coated with a capture antibody which binds or adsorbs to the plate in the solid phase. Blocking buffer is added to block nonspecific binding sites on the plastic. Cell culture supernatants containing unknown quantities of the analyte of interest are added along with the standards of known quantities. The analyte of interest then binds to the capture antibody. The plate is then washed and the detection complex composed of an enzyme linked to a detection (or secondary) antibody applied to the plate. After further washing to ensure that unbound antibody-enzyme conjugates are removed an enzyme substrate is added. The enzyme converts a typically colourless substrate to a coloured one that can be detected as an optical density reading. A standard curve yields an optical density for a known amount of analyte and the concentrations in the samples can be calculated from the optical density.

Diagram adapted with permission from Beckman Coulter products (www.microscopesblog.com/.../ELISA-710650.jpg)

2.1.12.2 ELISA methodology

Cytokine concentrations in supernatants from study consortium and MNC co-cultures were determined using commercially available ELISA kits according to the

manufacturers' instructions. The methodology for all assays was essentially the same with minor variations mainly in sample dilutions and depending on the manufacturer of the kit. Briefly, half area 96 well plates (Greiner Bio-one, Germany) were coated with 50µl of capture antibody prepared in coating buffer (PBS, Invitrogen; or 0.05M carbonate-bicarbonate buffer, pH 9.6, Sigma) and incubated overnight at 4°C. After discarding excess coating antibody, non-specific reactivity was blocked by incubation for 1 hour at room temperature with 1% bovine serum albumin (BSA; Sigma) in PBS (150µl/well). ELISA plates were then washed three times with wash buffer (1% BSA in PBS with 0.05% Tween-20 (Sigma); 200µl/well) before the sample or standard was applied in duplicate (50µl/well). After 2 hour incubation at room temperature, the plates were washed four times with the wash buffer as before and then the biotinylated detecting antibody was added (50µl/well) and incubated at room temperature for 1 or 2 hours, depending on the assay. Following this the plates were washed four times and avidin-horseradish peroxidase was added (50µl/well) and incubated for 20 or 30 minutes at room temperature (timings were assay dependent). Upon completion of the incubation with avidin-horseradish peroxidase the contents of the wells were discarded and the plates washed six times with wash buffer as before. Substrate chromogen (tetramethylbenzidine, BD Biosciences; 50µl/well; prepared according to the manufacturer's instructions) was then added and the blue colour allowed to develop (variable times for each assay). The reaction was stopped with 1M H_2SO_4 and the subsequent colour intensity (yellow) was recorded as the optical density at 450nm using a POLARstar Omega plate reader (BMG Labtech, Germany).

For the assay to be quantitative, a standard curve must be prepared for each assay as this enables cytokine concentrations in the samples to be calculated (Excel, Microsoft Office 2007). Standard curves were prepared by plotting concentration on the y-axis against optical density at 450nm (OD450nm) on the x-axis using Excel (Version 2007). An example is shown (Figure 2.10). Cytokine concentrations in the samples were calculated from the standard curve and corrected for any dilution made. Calculations were made using the equation of the line generated in Excel (Figure 2.10) as a value for x, i.e. the optical density value obtained, was available for all samples enabling calculation of the unknown, y, i.e. concentration of the cytokine of interest.

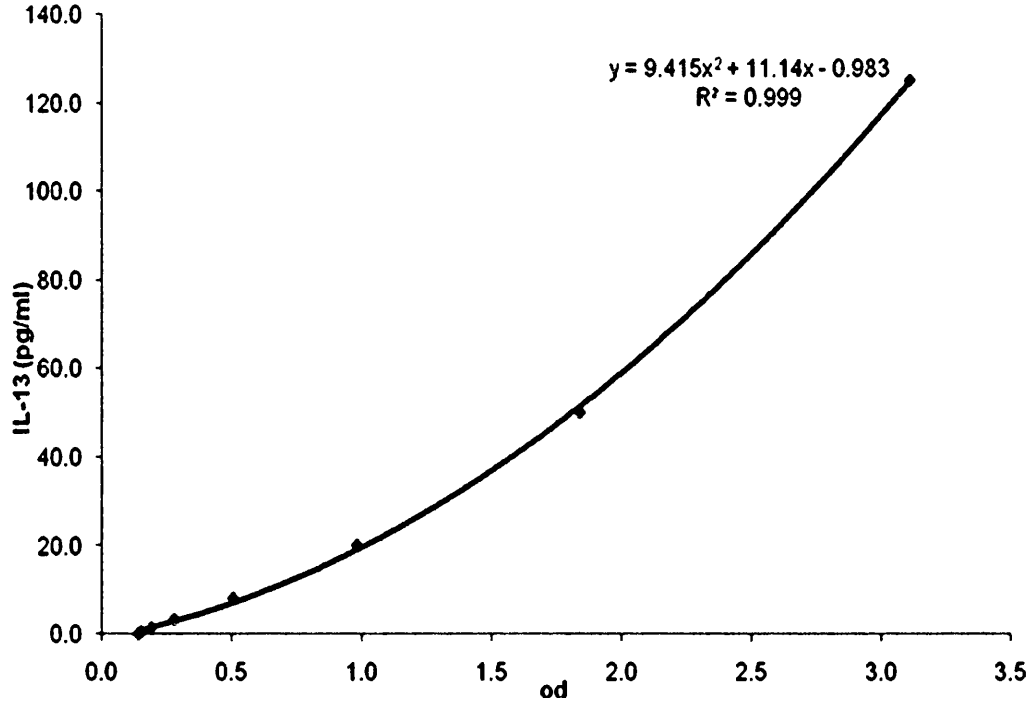


Figure 2.10: Example of a standard curve prepared during analyses used in this thesis.

Table 2.1: Details of ELISA kits used for the specific cytokines analysed for this study.

Cytokine	Sensitivity	Company
IL-12p70	15.6 pg/ml	OptEIA, BD Biosciences
IL-6	4.8 pg/ml	OptEIA, BD Biosciences
IL-1β	15.6 pg/ml	OptEIA, BD Biosciences
IL-10	7.8 pg/ml	OptEIA, BD Biosciences
IL-13	0.5 pg/ml	Pelikine Compact™ Sanquin, Amsterdam.
TNF-α	15.6 pg/ml	OptEIA, BD Biosciences
IFN-γ	4.7 pg/ml	OptEIA, BD Biosciences
TGF-β1	5.9 pg/ml	Opt EIA, BD Biosciences
IL-17	15.6 pg/ml	Duoset, R&D Systems

2.1.13 Statistical analysis

The student's t-test was used for the statistical analysis to determine whether cytokine responses to the study consortium could be deemed statistically significant. $P < 0.05$ was considered statistically significant. All data was expressed as mean ± SEM. Further details of the statistical analysis can be found in the relevant chapters.

2.2 Materials and methods for *in vivo* analysis - The PROBAT trial: probiotics for the prevention of allergic disease

2.2.1 Background to the study

The PROBAT trial is a randomised-double blind placebo controlled study conducted in South Wales that has been underway since 2004. The principal aim of this study was to investigate the immunomodulatory effects of a consortium of potential probiotic strains of Lactobacillus and Bifidobacterium species. The potential role of these commensal organisms in regulating immunity involved in the attenuation of the onset of IgE-mediated allergic disorder was the main focus of the study. The experimental approach adopted was one of probiotic supplementation to pregnant women from 36 weeks of pregnancy until the birth of the baby and the newborn from birth until 6 months of age. Infants were to be predominantly at high risk for the development of allergic disease. High risk was classified as those born into a family with a history of atopic disease: mother and/or mother's existing children and/or the father of current pregnancy have one or more of the following medically diagnosed IgE-mediated allergic diseases: asthma, atopic eczema or allergic rhinitis.

Consideration of the impact of probiotics on immune function in this age group was made by extensive laboratory based analysis of samples collected at the birth of the baby (umbilical cord blood and placenta), breast milk at 2 weeks and 6 weeks postpartum and blood from the infant at 6 months of age. Faecal samples were collected throughout to monitor colonisation with the species present in the study capsule. The potential impact of the probiotic consortium used on immune function was further explored by the inclusion of infants from low risk families; defined as those who had no first degree relatives with an atopic disorder.

The principal outcomes of the PROBAT trial were four fold. Can perinatal administration of the potential probiotic consortium of choice (see 2.1.1):

1. Reduce the incidence of IgE-mediated atopic disease during infancy and childhood?
2. Reduce the prevalence of eczema during the first year of life?
3. Reduce the prevalence of asthma at 5 years of age?

4. Regulate key immune parameters which could potentially attenuate the risk of IgE-mediated allergy in later life?

2.2.2 Power analysis

The PROBAT trial follows on from an innovative study conducted in Finland demonstrating that nearly half of the infants perinatally exposed to probiotics developed eczema in the first two years of life compared with those exposed to a placebo (Kalliomaki *et al.* Lancet 2001 and 2003). The frequency of atopic eczema in the probiotic group was half that of the placebo group: 23% versus 46%. For the power calculation of the study in Swansea, it was assumed that forty percent of infants in the placebo group would develop eczema by age 6 months. A total of 236 infants would allow the detection of a reduction of eczema in the probiotic group to 20% with 90% power at the 5% level of statistical significance.

Demographics and size of the study population

We enrolled 454 allergic women delivering in the South Wales, Swansea city area between Month 2005 and Month 2009 from a total of 1419 pregnant women approached and assessed for eligibility. Of these, 220 were randomized to receive the study consortium and 234 to receive the placebo. Allergic women were preferentially recruited to the PROBAT trial as their unborn children were considered to be at high risk of developing atopic disorder in 91% (n = 413) of recruited women.

The study was powered to evaluate probiotics in the prevention of asthma and we expected 21% in the placebo group to develop asthma by age 5 years. A total of 540 infants (270 in each arm) would have allowed the detection of a reduction of the frequency of asthma of 50% in the intervention group with 90% power at the 5% significance level. We expected that 40% of infants in the placebo group would have eczema by age 2 years (Kaur *et al.* 1998; Wadonda-Kabondo *et al.* 2003).Therefore, a total of 308 infants (154 in each group) would be sufficient to detect a 50% reduction in eczema frequency (20%) in the probiotic group with 90% power at the 1% significance level. Recruitment began in May 2005 but, because of funding constraints, was terminated early. At that point, 402 mothers had been recruited and the last infant was reviewed at the 2 year follow-up visit in November 2009. A statistical power analysis to determine the number of subjects needed to offset the expected heterogeneity of the immune response in the birth cohort was conducted.

Based on previous studies by Kalliomaki *et al.* (2001) the numbers recruited in the study allow for detection of differences in immunological responses between the groups at a statically power of over 95% for all cytokines).

Maternal allergy was defined as a doctor-diagnosed clinical history of asthma, allergic rhinitis or eczema and a positive skin prick test (SPT) to one or more common allergens (HDM; grass, pollens, cat pelt, dog hair, feathers, moulds, and cockroach extracts). A weal size of 3mm above the negative control was considered positive. Additionally there were no statistically significant differences between the probiotic and placebo groups with respect to gender, gestational age, birth weight, older siblings, day care attendance, or exposure to smoking at home.

Immune response in a large birth-cohort will show marked heterogeneity attributed to factors such as health status, genetic variability, diet, maternal smoking, level of physical activity, or cognitive status .

Confounding variables that could affect the integrity of the current study are briefly described below based on the findings of (Gardner and Murasko 2002).

Health Status of the Subjects

Since subjects recruited on the PROBAT trial were specifically selected on the basis of an increased genetic predisposition to developing atopy in later life. To delineate changes in immune function due to age, expression of atopic disease or a specific effect of the study consortium during the supplementation period all adverse events effecting immune status that could potentially skew experimentally analysed *ex vivo* immune responses were recorded in (Allen *et al.* 2010). This step was of considerable importance since it is widely reported that application of antibiotics to resolve early childhood infection is linked with expression of atopic disorder in later life (Almqvist *et al.* 2012).

Supplementation Group allocation occurred by block randomization, with stratification according to maternal allergy (asthma vs. other allergy), parity (first child vs. two or more children) and paternal allergy (allergic vs. non-allergic). Infants

allocated to the study consortium received 1×10^{10} of the bacteria strains in maltodextrin (Cultech) daily, while those in the control group received maltodextrin alone. The products were matched in taste and appearance. Supplements were supplied in capsules administered daily to lactating mothers for 36 weeks gestation and then to their newborns for the first 6 months of life, independent of feeding methods. The consortium conformed to the FAO/WHO expert panel guidelines for probiotics (resistance to acid and bile; adherence to cells of the intestinal epithelium and colonization in the intestinal tract; antagonistic activity towards enteric pathogens and maintenance of strain identity and viability throughout shelf-life).

2.2.3 Ethical approval

Ethical approval was obtained from the local research ethics committee, LREC for Bridgend, Neath Port Talbot and Swansea in February 2004 (International standard randomized controlled trial, ISRCTN 26287422). Informed written consent was obtained from all pregnant women recruited to the study (Appendix 2 for patient information sheet and consent form). Ethical approval was granted and the reference number for this study is 2004.02

2.2.4 Study participants

Women aged 16yr or older with a normal singleton pregnancy attending antenatal clinics in hospitals or general practice surgeries in the greater Swansea area were eligible to join the study. The majority of women recruited to the study were carrying a fetus at increased risk of atopy, defined as a fetus with a first-degree relative with a familial history of atopy: mother, father or sibling with either asthma or eczema diagnosed by a health professional or allergic rhinitis treated by a doctor. A group of women pregnant with a fetus at low risk of atopy and atopic disease by virtue of an absence of a first degree relative with asthma, eczema or allergic rhinitis as defined above were also recruited

2.2.5 Recruitment to the PROBAT trial

Pregnant women were invited to join the study before 35-36 weeks gestation during the booking visit (usually around 10 to 16 weeks of pregnancy) or at subsequent routine antenatal clinic visits with the local NHS trust. Women were initially given information explaining the aims and designs of the study and verbal consent was

sought to confirm willingness to further consider participation in the study. The women were provided with more information about the study and if they were willing to progress with the study, informed written consent was obtained. At approximately 36 weeks of pregnancy women who had consented were visited at their homes or in hospital clinics by a member of the research team. During the visit the aims and design of the PROBAT study were reviewed and the inclusion and exclusion criteria were explained. Women were invited to provide a blood sample at the 36 week routine antenatal clinic visit.

2.2.5.1 Inclusion criteria

- Mother aged ≥ 16 years.
- Scheduled to give birth at Swansea Singleton Hospital.
- Gestation at delivery >36 weeks.
- Freely given, signed, informed consent to participate in the randomised-blind placebo controlled trial.

2.2.5.2 Exclusion criteria

- Any adverse medical condition likely to affect the outcome of the pregnancy.
- Any adverse medical condition affecting the mother or newborn.
- Member of the household or infant's sibship already recruited on the study.
- Women unwilling to discontinue use of other bacterial dietary supplements.

2.2.6 Random allocation and blinding

As a randomised double blind placebo controlled study, neither the mother nor any member of the research team knew which preparation a particular mother and baby was taking throughout the study until at least the time of writing this thesis. This was a requirement that has been adhered strictly to, ensuring that a comprehensive, unbiased and fair assessment could be made as to whether or not the probiotics have been effective in preventing the development of atopy/atopic disease. A computer-generated, random allocation sequence was generated by the independent statistician (Duolao Wang) to enable apportioning of each mother-infant dyad to either the study consortium or placebo arm of the study on a 1:1 basis.

Probiotic and placebo powders were prepared by Cultech Ltd and the random allocation sequence for the probiotic and placebo vials was maintained by the

company: a series of cards bearing the randomised study identification numbers in opaque envelopes were prepared. Following informed consent, mothers were allocated the next card in the series and thereby allocated randomly to either the probiotic or placebo group. Probiotic and placebo preparations were identical in appearance therefore neither the participants nor the study team were able to distinguish probiotic from placebo. Cultech Ltd ensured that the release of the probiotic or placebo allocation code was made available to the academic research staff only after the compilation of the 2 year clinical data and immunological information generated from cord samples collected around the birth of the baby and at 6 months of age. Access to the randomisation code was provided to the local NHS Trust so that it was available if any mother or baby on the study presented to one of the local hospitals in an emergency.

2.2.7 Intervention

The probiotic and placebo products used in the PROBAT study were prepared according to stringent manufacturing processes. The raw materials and final products were subject to robust microbial quality control testing to ensure that each of the component micro-organisms met the standard required specification and identity and that no contaminating organisms were present in the probiotic capsule. Women during the last month of pregnancy and their infants from birth to age 6 months received daily vegetarian capsules composed of hydroxypropyl methylcellulose containing either the probiotic supplement or the placebo.

2.2.7.1 Constituents of the probiotic supplement

Probiotics were manufactured as a freeze-dried powder, in a vegetarian capsule and contained in vacuum sealed bottles. Each 100mg of powder contained a diverse consortium of potentially probiotic organisms. The probiotic preparation contains live bacteria and the bacterial strains and numbers of organisms taken per day were: 2 live strains of Lactobacillus and 2 live strains of Bifidobacterium. In total 1×10^{10} CFUs of probiotic bacteria (See 2.1.1) was administered each day to study participants.

2.2.7.2 Constituents of the placebo

The placebo comprised an equal volume of inert maltodextrin powder identical in appearance to the probiotic preparation.

2.2.8 Method of probiotic administration

To maximise probiotic colonisation within the intestinal tract of the newborn, participating mothers were required to take the study preparation once a day from 36 weeks gestation until delivery of the baby. From the day of delivery until age 6 months, mothers were expected to administer the same dosage of the preparation to their infants. Mothers were visited every 6 weeks by a member of the research team to ensure a fresh supply of the preparations if required and to answer any pertinent questions regarding the study and also to retrieve the empty vials as a measure of compliance.

2.2.8.1 Administration to the pregnant woman

Participating women were randomly allocated to take either the active probiotic preparation or an identical placebo (See 2.2.6). The expectant woman was expected to take one capsule per day of the freeze dried powder as a food supplement sprinkled onto meals from 36 weeks of pregnancy until delivery.

2.2.8.2 Administration to the infant

Participating women were expected to administer the contents of one capsule per day of the trial supplement as soon as possible following the birth of their child every day from birth to 6 months of age. The trial capsule was to be split open and administered to their newborns via one of the following methods:

- Sprinkling the freeze dried powder directly onto the mother's nipple;
- Sprinkling powder directly into the newborns mouth;
- Mixing the powder in a cup with a small amount of expressed breast milk or clean water prior to administering to the newborn;
- For bottle fed babies, the powder could be added directly to the milk or water.

Mothers were strongly urged to breastfeed in accordance with accepted postnatal ward practice and were encouraged to breast feed during the course of supplementation due to the well-known and widely reported health benefits of breastfeeding for both mother and child.

2.2.9 Arrival of expectant woman onto antenatal and labour wards and collection of samples

Expectant women participating on the PROBAT trial could be identified by a trial label on the front of their patient notes. Midwives employed by the trial ensured that participating women were carrying a supply of the trial supplement for themselves and their soon to be newborn as part of their personal belongings. At delivery, umbilical cord blood and the placenta/attached membranes was collected for use as described in section 2.3. Delivery suite staff then contacted the study team to notify them of samples for collections.

2.2.10 Out of office hours

To cater for babies being delivered out of office hours, an on-call system was utilised - each member of the research team was allocated a weekend on the on-call rota and carried the study mobile phone to ensure that samples collected during the weekend could be collected and processed in the laboratory.

2.2.11 Clinical outcomes

All participants were visited at home every 6 weeks until the baby was 6 months old to provide supplies of the study preparations and to monitor compliance.
Assessment of the trial clinical outcomes was performed by a trained member of the research team at 6 months and 2 years of age (to date; further follow-up is planned).
At 6 months of age, the assessment involved:
(i) a detailed examination of the child to assess the severity of atopic eczema defined according to the presence of specific symptomatic manifestations including itchy, inflamed rash affecting the face, scalp or extensor surfaces/flexures of the limbs;
(ii) Parents were asked to complete a questionnaire regarding symptoms of allergy and general wellbeing;
(iii) Skin prick testing (See 2.2.10.2);
(iv) Peripheral blood sample collected for laboratory analysis (See section 2.3.6).

The infant's health records held by the general practitioner also were assessed by health professionals to identify clinical diagnoses of atopy. Blood collection from the infant was subject to parental consent.

2.2.11.1 Procedure for allergy assessment in newborns

A trained member of the research team was designated to ask questions regarding the general health of the participating mother in terms of the prevalence of allergic disorders in the family and also about the families exposure to environmental factors linked with allergy such as keeping domesticated pets and use of antibiotics. Newborns were visited every 6 weeks to the age of 6 months and every year to the age of 2 years to collate information assessing the current atopic status.

2.2.11.2 Skin prick tests

Skin prick tests (SPTs) were performed at the ages of 6 months and 2 years and involved placing on the skin a drop of fluid containing one of a number of common allergens including β-lactoglobulin, ovalbumin, *Dermatophagoides pteronyssinus*, cat dander, and grass pollen. The skin is then gently scratched through the drop and the subject observed to determine whether a local skin reaction ensues. The allergic skin reaction usually reflects atopic sensitisation to a specific allergen in the fluid and is represented as flare and wheel. A positive (histamine) and negative (diluent) control is always included.

2.2.12 Additional information

In order to interpret the findings of the PROBAT trial, additional information regarding possible risk factors for IgE-mediated allergy where collected from all participants and analyzed by virtue of a questionnaire:

- Brief details of atopic diseases in first degree relatives
- Number of older siblings
- Household pets
- Maternal smoking during pregnancy and exposure to cigarette smoke in the home
- Mode of delivery
- Method of feeding
- Attendance at nursery
- Drugs received during pregnancy, labour, infancy and childhood, especially antibiotics

2.2.13 Potential side effects of probiotic supplementation in the newborn

Probiotics are food supplements "generally regarded as safe" according to the recommended classification by the Food Standards Agency. A recent publication form our research group indicates that dietary supplementation with lactobacilli and bifidobacteria is well tolerated and not associated with adverse events during late pregnancy and early infancy (Allen *et al.* 2010).

2.3. Procedures for PROBAT trial sample collection

Additional *ex vivo* laboratory-based analyses were conducted using samples collected during the PROBAT study to elucidate the potential immunomodulatory effect of the study consortium. The following describes the samples collected and what they were used for.

2.3.1 Maternal blood sample at 36 weeks of gestation

At the time of performing this study, pregnant women under the care of the local trust routinely had blood taken at around 36 weeks of pregnancy as part of their clinical obstetric care. Women who consented to be involved in the PROBAT study and had this routine sample take also provided a peripheral blood sample for this study: 9ml into sodium heparin (Vacutainer, Greiner Bio-One); 4ml into gel and clot activator tubes (Vacutainer).

2.3.2 Umbilical cord blood at birth of the baby

Umbilical cord blood samples were collected routinely immediately following the birth of the newborn child and preceding the cutting of the umbilical cord. Blood was collected into sodium heparin and gel and clot activator tubes as for maternal sample.

2.3.3 Placenta at birth of the baby

Placentas were collected from the hospital and the time elapsed between delivery and arrival in the laboratory recorded. The placenta was weighed and if not longer than 2hr had passed since delivery it was processed as described below.

2.3.4 Breast milk

Breast milk samples from breast feeding mothers at 2 weeks and 2 months after the baby's birth where collected. Samples were aliquoted in a Class II tissue culture cabinet to limit contamination. Up to 5 aliquots of approximately 1ml breast milk were transferred to microfuge tubes using a sterile pastette and immediately stored at -80°C.

2.3.5 Faeces

Faecal samples were obtained from the infants on this study to determine whether probiotic bacteria could be recovered more frequently in the stools. Stool samples were collected from infants to assess the faecal microbiota to determine differences in probiotic colonisation between the placebo group and intervention group. Samples were collected just after birth and at 3 months, 6 months and at regular intervals thereafter to trace the duration of colonisation after stopping administration. These samples were analysed by Cultech Ltd to avoid unmasking of the research clinicians regarding the allocation sequence. An aliquot of these stool samples were frozen and stored for future measurement of non-invasive markers of gut inflammation and permeability.

2.3.6 Blood from infant at 6 months of age

Blood samples (up to 5ml venous blood into sodium heparin) were obtained by trained paediatricians from veins in the back of the hand or antecubital fossa using an aseptic technique and 23 gauge butterfly needles after applying a topical anaesthetic cream.

2.4. Procedures for PROBAT trial sample analysis

2.4.1 Analysis of umbilical cord blood samples

Umbilical cord blood was used as follows:

Heparinised sample: automated haematology analysis (n = 100); whole blood culture (n = 108); whole blood flow cytometry (n = 63); sample for DNA analysis (n = 212); preparation and culture of MNCs (n = 102); plasma (n = 197). Clotted sample was used for the preparation of serum (n = 226).

2.4.1.1 Automated haematology analysis

Total blood counts and differential were measured using a CellDyn® 3200 analyser (Abbott Diagnostics, Germany). CellDyn® is a multi-parameter automated haematology analyser designed for *in vitro* diagnostic use in clinical laboratories. Further details are provided in Chapter 6.

2.4.1.2 Whole blood culture

Whole blood cultures were used to study key pro- and anti-inflammatory cytokine responses in the presence of the inflammatory stimuli such as lipopolysaccharide (LPS) and peptidoglycan (PGN). Umbilical cord blood (UCB; 200 µl) was diluted 1 in 4 with 600 µl of culture media (RPMI1640/Glutamax with 0.5 mM 2-mercaptoethanol, all Invitrogen) in 4.5 ml culture tubes (Greiner Bio-one). Duplicates were prepared. The following stimuli were used: unstimulated, LPS (10ng/ml; Invivogen), PGN (3µg/ml; Invivogen), IFN-γ (10ng/ml; Invitrogen), LPS + IFN-γ, PGN + IFN-γ. IFN-γ was added to appropriate tubes and all tubes were left to incubate for 90 minutes at 37°C and then LPS or PGN added as appropriate. All tubes were then left to incubate at 37°C in 5% CO_2-in-air for 20 – 22 hours. Cell free culture supernatants were harvested by centrifugation and then supernatants were transferred to labelled microrack tubes (Greiner) and stored at -80°C until analysis using ELISA.

2.4.1.3 Mononuclear cell culture

Isolated mononuclear cells (See section 2.1.3) were placed into 4.5ml culture tubes (Greiner Bio-one, Germany) containing 500µl media (AIMV serum free medium supplemented with 0.5mM 2-mercaptoethanol; all Invitrogen) in aliquots of $0.5x10^6$ cells/500µl. Once the cultures were prepared, two tubes were left un-stimulated, two were treated with the PHA (1µg/ml; Sigma) or, if cell numbers permitted, SEB (200ng/ml; Sigma) or anti-CD3/CD28 T cell expansion beads (5µl/500µl; Dynal, Invitrogen). All tubes were incubated at 37°C in 5% CO_2-in-air for 48 hours. Cell free culture supernatants were collected by centrifugation and stored at -20°C until analysis using ELISA.

2.4.1.4 Whole blood flow cytometry

Multi-parameter flow cytometry was used to assess various leukocyte cell subpopulations. Cord blood collected into sodium heparin was used and immunophenotyping done using a whole blood lysis method (Perez *et al.* 2005; Schwonzen *et al.* 2007). Principally whole blood was incubated with monoclonal antibodies; the stained sample was treated with a lysing solution to remove red blood cells while preserving the leukocytes for flow cytometric analysis. Predetermined concentrations of antibodies were added to FACS tubes and then 50µl of cord blood was added to each tube, vortexed and incubated for 30 minutes on ice in the dark. Lysis of red blood cells was then performed - 3mls of FACS Lyse (BD Biosciences) was added to each tube which was then vortexed and incubated for 10 minutes in the dark at room temperature. Tubes were then centrifuged at 4°C, 510 x *g* for 7 minutes, supernatant discarded, and 3mls of FACS buffer (PBS/0.2% BSA/0.05% sodium azide) added. Following repeat centrifugation and discarding the supernatant, 400µl of FACS fix (BD Cell Fix, BD Biosciences) was added. Samples were covered and then stored in the fridge before acquisition within 24 hours using the BD FACSAria™ flow cytometer and CellDiva Software (BD Biosciences, San Jose, CA). The panel of monoclonal antibodies and fluorochromes used are listed in Table 2.2.

Table 2.2: Panel of fluorochrome-conjugated monoclonal antibodies and isotype controls used for whole blood samples.

Antigen	Fluorochrome	Isotypes	Clone	Source
CD3	PE/APC-Cy7	mIgG2a	S4.1	Invitrogen (Caltag)
CD4	APC/APC-Cy7	mIgG2a	S3.5	Invitrogen (Caltag)
CD8	Pacific Blue	mIgG2a	3B5	Invitrogen (Caltag)
CD25	PE	mIgG1	3G10	Invitrogen (Caltag)
CD45RA	FITC	mIgG1	L48	BD Biosciences
CD45RO	APC	mIgG2a	UCHL1	BD Biosciences
CD45	Pacific Blue	mIgG1	H130	Invitrogen (Caltag)
CD14	FITC	mIgG2a	TüK4	Invitrogen (Caltag)
CD5	FITC	mIgG1	CD5-5D7	Invitrogen (Caltag)
CD16	Alexa 647	mIgG1	3G8	BD Biosciences
CD19	PECy5.5	mIgG1	SJ25-C1	Invitrogen (Caltag)
CD21	PE	mIgG1	BU32	Invitrogen (Caltag)
CD23	FITC	mIgG3	Tü1	Invitrogen (Caltag)
CD69	PECy5.5	mIgG2a	CH/4	Invitrogen (Caltag)
CD123	PE	mIgG1	9F5	BD Biosciences
HLA-DR	PECy5.5	mIgG2b	Tü36	Invitrogen (Caltag)
HLA-DR	PerCP	mIgG2a	L243	BD Biosciences
Lineage*	FITC	mixed	mixed	BD Biosciences
Isotype control	various	mIgG1	MOPC-21	BD Biosciences
Isotype control	various	mIgG2a	no data	Invitrogen (Caltag)
Isotype control	various	mIgG2b	no data	Invitrogen (Caltag)
Isotype control	FITC	mIgG3	no data	Invitrogen (Caltag)

2.4.2 Placental explant culture

Placental explants cultures (0.2g of wet tissue/ml of media) were prepared as described (Hanna *et al.* 2000). Briefly, the overlaying deciduas basalis on the maternal side of the placenta was removed and $1cm^3$ pieces of placental tissue were taken from different sites across the placenta and placed into sterile PBS. Disturbance of the chorioamnion was avoided. Placental tissue was washed repeatedly with PBS to remove contaminating blood, and fresh PBS was used for washing steps. The tissue was then minced into smaller pieces (approximately 1-2 mm^3) and washed further with PBS. Pieces of placental tissue (0.5g) were transferred into each well of a standard tissue culture 6-well plate (Greiner Bio-one, Germany) containing 2.5ml of Ultraculture medium (Cambrex, Belgium) supplemented with 2mM Glutamax (Invitrogen, UK) and 100U/ml penicillin G, 100µg/ml streptomycin sulphate and 0.25µg/ml amphotericin B (PSF; Invitrogen, UK). Care was taken to avoid any blood clots or fibrous tissue. Four wells of a 6-well plate were left unstimulated and two were stimulated with LPS (10ng/ml) wells. Plates were then incubated at 37°C in 5%CO_2-in-air for 24 hours. Tissue free culture supernatants were collected by centrifugation and stored at -20°C until analysis by ELISA. Extreme care was taken to limit LPS/endotoxin contamination during explant preparation, including the use of disposable plastic-ware and other consumables (e.g. scissors) whenever possible (Jones *et al.* 1997). All media/reagents were tested by the manufacturers and found to be endotoxin free.

2.4.3 Analysis of blood from 6 month infant

Infant blood was used as follows:

Heparinised sample: automated haematology analysis (n = 158); whole blood culture (n = 107); whole blood flow cytometry (n = 80); preparation and culture of MNCs (n = 96); red blood cell pellet (n = 152); plasma (n = 181).

2.4.3.1 Automated haematology analysis

As for umbilical cord blood with the exception that the sample could be diluted in PBS prior to analysis if samples size was limiting. Values used in analysis were corrected for this dilution.

2.4.3.2 Whole blood culture

As for umbilical cord blood.

2.4.3.3 Mononuclear cell culture

As for umbilical cord blood.

2.4.4.4 Whole blood flow cytometry

As for umbilical cord blood.

2.4.4 ELISA analysis of supernatants from whole blood and MNC cultures

Whole blood culture supernatants were analysed for: TNF-α and IL-10 for unstimulated and LPS- and PGN-treated samples; IL-12p70 for unstimulated and LPS- and PGN-treated samples in the presence and absence of IFN-γ. Mononuclear cell supernatants were analysed for: IL-4, IL-10, IL-13 and IFN-γ. These methods are described in further detail in (4.2.1 to 4.2.2)

2.5 Samples archived for future work

Samples stored for future work at a later stage are described below.

2.5.1 Placental biopsies for RNA analysis

Additional placental tissue (3 pieces) was removed and washed several times in cold PBS to remove contaminating blood. A fixed amount of tissue (0.2-0.26g) was transferred into 1.3ml of RNAlater™ (Sigma, USA). Biopsies in RNA later were stored at 4°C for 48 hours and then transferred to -80°C for long term storage.

2.5.2 Sample collection for DNA analysis.

Aliquots (200μl) of whole anti-coagulated umbilical cord blood were placed in labelled tubes and stored at -80°C.

2.5.3 Preparation of plasma

Plasma was kept following the density gradient centrifugation process (as previously described 2.1.3) and transferred to a bijou. The plasma sample was sterile filtered (0.22μm; low protein binding polyesthersulfone filter; Millipore, Bedford, MA, USA) and aliquoted into well-labelled tubes for storage at -80°C until needed.

2.5.4 Preparation of serum.

Umbilical cord blood collected into the yellow-top tube (gel and clot activator tube; Vacutainer, Greiner Bio-one) was used for the preparation of serum. The sample was left at room temperature for at 60 – 90 minutes to clot, centrifuged at 1800 x *g* at room temperature for ten minutes, and stored in labelled aliquots at -80°C until needed.

2.6 Statistical analysis

Principle outcomes measured by the PROBAT trial directly concerned with this thesis were: the impact of maternal probiotic consumption during the last few weeks of pregnancy on immune function at birth; and the impact of infant probiotic consumption for the first 6 months of life on immune function at 6 months of age. Due to the varied and/or detailed nature of the statistical approaches used for analysis of *in vitro* and *ex vivo* experiments these are detailed in the relevant chapters.

Chapter 3: Effects of study consortium on cytokine production from umbilical cord blood (CBMCs) and adult peripheral blood mononuclear (PBMCs) cells *in vitro*

3.1 Background

Perinatal probiotic supplementation of newborns with a familial history of allergy has become a rationale of increasing research interest to prevent or attenuate the symptoms of IgE mediated atopic disorder. This rationale is in part due to the well documented immunomodulatory effects of probiotic bacteria in human immunity, and has generated an avenue for development of clearly defined *in vitro* models representing the newborn immune response to probiotic bacteria. Since data modelling the immunomodulatory impact of probiotic bacteria upon the cellular constituents of newborn immunity *in vitro* is scarce, the use of mononuclear cells prepared from umbilical cord blood (CBMCs) isolated by density gradient centrifugation (See Chapter 2) was at the heart of the *in vitro* model employed in this thesis. The principle focus of chapter 3 was to therefore provide *in vitro* immunological data supporting the application of probiotic bacteria in the PROBAT trial, and to provide the clinical framework for experimentally driven hypotheses into how perinatal probiotic supplementation might positively modulate early newborn immunity at the cytokine level, particularly in clinically predisposed atopic individuals. The general thesis hypothesis is that, immunomodulation mediated by the consortium might positively influence and divert the deleterious immune equilibrium rendering a child atopic in later life, (See Chapter 1) towards a more balanced scheme of immunity as seen in non-atopic individuals.

Information gathered from this *in vitro* study was required to support an assessment on whether perinatal probiotic supplementation using the consortium could be efficacious against the onset and/or progression of IgE-mediated atopy in later life. Furthermore, *in vitro* data produced at the cytokine level essentially provided the

foundation for experimentally driven hypotheses into how the consortium may function *in vivo* during development of early newborn immune mechanisms necessary to counter the onset of IgE mediated atopy in later life (See chapters 4, 5 and 6).

Since perinatal supplementation to prevent allergic disease development is a field of increasing interest, the use of cord blood mononuclear cells (CBMCs) in these models is essential. There is a paucity of research considering the immunomodulatory effects of probiotic microorganisms using CBMCs to model the neonatal immune response. A perinatal supplementation study employing the study consortium on infants at birth and 6 months of was initiated in Swansea in 2005. This offered the opportunity to concurrently study the effects of the same four micro-organisms on immunological phenotypes measurable *ex vivo* using CBMCs and peripheral blood at 6 months of age versus those generated in an *in vitro* model using CBMCs.

The strains of probiotic used are postulated to orchestrate key immunomodulatory actions upon host immunity characterised by generation and maintenance of important activities of mucosal immunity such as: anti-microbial and pro-inflammatory actions mediated by Th1 type cytokines (IL-12, TNF-α and IFN-γ); anti-inflammatory and oral tolerance activities, induced and maintained by immunoregulatory cytokines (IL-10 and TGF-β); stimulation of adaptive immune responses marked by local and systemic IgA and IgG determined by the Th2 type cytokines (IL-4 and IL-5) (Brandtzaeg 2002; Brandtzaeg 2009). Cytokine synthesis patterns from haematopoietic cells induced by LAB *in vitro* and *in vivo* are strain specific (See Chapter 1 section 1.7.2).

In order to interpret how CBMCs might respond to a consortium of potential probiotic microorganisms it is imperative to gain an appreciation of the nature of newborn immunity in terms of the maturational stage of the developing immune system and how it differs from adult immunity. After delivery, the immune system of the neonate is immature and undergoes rapid development strongly influenced by the quality of immune education provided by antigenic stimuli encountered during the initial years of life. The immune system of the growing fetus fundamentally develops towards a Th2-skewed in healthy infants in which Th2 cells predominate (Prescott *et al.* 1998). However, newborns generally have a reduced ability to produce many

cytokines and growth factors compared to the adult. Passive transfer of maternal antibodies across the placenta from mother to child facilitates this fetal predisposition in favour of Th2 humoral immunity as opposed to Th1 cell mediated immunity. Newborns are initially protected by these maternal antibodies for the first two to three months of life, and are relatively free of bacterial infections during this time (as reviewed by (Jordan *et al.* 2008). Antibody production does not reach adult levels until age four years. NK and cytotoxic T cells have an attenuated function in the neonate to limit exuberant and potentially toxic Th1 immune responses. Consequently newborns are somewhat vulnerable to viral and intra-cellular infections (Jordan *et al.* 2008).

3.1.1 Cytokines to be studied

The ideal cytokines to be studied are the Th2 cytokines postulated to have a mechanistic role in atopic disorder and/or those demonstrated to be modulated by probiotics from previous studies such as the Th1 cytokines. There is considerable overlap between the cytokines of interest in both development and maintenance of allergic disease and those demonstrated by other investigators to be up or down-regulated in the presence of probiotic bacteria. An overview of each cytokine chosen for inclusion in this investigation was provided in terms of their induction by probiotic bacteria and their role in allergy (See 1.3.6 to 1.3.8).

3.1.2 Need for the study

The *in vitro* immunomodulatory attributes of a consortium of potentially probiotic lactobacilli and bifidobacteria strains were characterised within a traditionally employed *in vitro* model used to determine how potentially probiotic strains indigenous to the intestinal microbiota influence innate and adaptive immunity. The conglomerate of strains employed are more accurately classified as potentially probiotic since they have not yet been confirmed in any therapeutic, clinical or supplemental study to have a beneficial effect on human health; therefore for ease and clarity the strains will be referred to as the study consortium.

3.1.3 Aim of study

The aim of this study was to gain an insight into the immunomodulatory mechanisms of action of the probiotic study consortium employed in the PROBAT trial by studying their interactions with mononuclear cells. The initial goal was to screen for cytokines produced by mononuclear cells prepared from umbilical cord blood (CBMCs) and adult peripheral blood (PBMCs) in response to the study consortium *in vitro*. The capacity of the study consortium to modulate newborn immunity at the cytokine level, and any differences in responses between CBMCs and PBMCs was also examined. Bearing in mind that newborns have a diminished functional immune capacity compared to the adult (See Chapter 1), we aimed to determine whether this is apparent in responses of CBMCs to the consortium and elucidate possible mechanism for any differences. Mechanisms by which the consortium generated interpretable signals to the immune system were also analyzed by defining the key cell types involved in cytokine production and whether this production is dependent on direct contact between mononuclear cells and the consortium.

3.2 Rationale and Methodology behind study

3.2.1 Viability of the study consortium

Since our proposed *in vitro* model was dependent on interactions between the consortium and mononuclear cells it was imperative that a viability assessment of the consortium in typical laboratory culture systems used for mammalian cells was first analysed. The lactic acid and bifidobacteria strains present in the study consortium are Gram-positive bacteria encapsulated in a freeze dried form. Since the focus of the present thesis was to analyse the immunomodulatory effects of probiotics on neonatal immunity, it was imperative that the viability of the probiotics strains in typical laboratory culture systems used for mammalian cells was first analysed. The present study sought to determine whether the study consortium remains viable in cell culture media used for culture of umbilical cord and peripheral blood mononuclear cells in ours and others research laboratories: serum free AIMV supplemented with 2-mercaptoethanol (complete AIMV) or RPMI1640/Glutamax supplemented with 5% FBS and 2-mercaptoethanol (complete RPMI all Invitrogen). Probiotic strains of the study consortium employed in the PROBAT trial were incubated in the presence of complete RPMI or complete AIMV over a 24hr period. The viability of organisms was assessed by monitoring total viable counts (TVCs; section 2.1.2).

3.2.2 Study consortium-mononuclear cells *in vitro* co-culture model

Although a system of studying direct interactions between cells of the mucosal immune system and the commensal bacteria in their natural GIT environment is laborious to replicate in laboratory practice, the use of mononuclear cell/haematopoietic cell-probiotic co-culture studies to model microbial host cell interactions *in vitro* offers a commonly acceptable alternative. The reliability of the *in vitro* model is supported by the fact that interactions between commensal bacteria and leukocytes during real time gastrointestinal physiology occurs via bacterial translocation through the epithelial barrier (Deitch *et al.* 1991; Sedman *et al.* 1994). It has been postulated that increases in non-specific (phagocytosis, NK cell activities) and specific (antibody synthesis) immune activities observed in mammalians can be attributed to increased translocation of probiotic bacteria through the epithelial barrier due to ingestion of probiotic strains of LAB origin (Miettinen 1996).

Mononuclear cells were prepared from anti-coagulated umbilical cord and adult peripheral blood by density gradient centrifugation (section 2.1.3). CBMCs or PBMCs were incubated with: (i) a dose course of study consortium for 24hr prior to harvesting of cell/bacteria free supernatants for later analysis using specific ELISAs for TNFα, IL-10, IL-1β, IL-6 and IL-12p70; (ii) 10^6 CFUs/ml of study consortium for 24hr prior addition of PHA (1μg/ml) or SEB (200ng/ml) for a further 24hr incubation at which time cell/bacteria free supernatants were harvested for later analysis using specific ELISAs for IFNγ, IL-4, IL-13 and IL-17.

3.2.3 Determining the principle cellular responders to the consortium

$CD14^+$ monocytes and $CD14^-$ depleted mononuclear cells were prepared using magnetic microbeads (Miltenyi Biotec) with purity/depletion monitored using flow cytometry. Contact between mononuclear cells and bacteria was abrogated by the use of trans-wells (section 2.1.5) and the cytokine response to 10^6 CFU/ml of the study consortium determined. For this part of the study only TNF-α was measured as an output.

3.3.1 Optimising viability of probiotic strains present in cell culture medium using traditional microbiological techniques

Optical density measurements (OD) were initially undertaken as a qualitative measure to determine the turbidity of liquid cultures containing lactobacilli and bifidobacteria strains of the study consortium maintained in either complete RPMI or complete AIMV. Changes in turbidity were used as a qualitative measure of bacterial proliferation. Measurement of turbidity in MRS broth served as a positive control as it is well documented that lactobacilli and bifidobacteria grow well in this media (Tannock 1999). This was confirmed in our study as strains proliferated in MRS; much more so than in either complete RPMI or complete AIMV (Figure 3.1). Figure 3.2 shows the growth in complete RPMI or complete AIMV with a different scale on the y-axis. From this it can be seen that a gradual increase in turbidity of a liquid culture occurs in complete RPMI with OD increasing from 0.046 to 0.056 and peaking at approximately 6 hours before declining slightly to 0.053 by 24 hours. Conversely, the turbidity change in complete AIMV was minimal with OD increasing from 0.046 to 0.047 by 6 hours before declining to 0.045 by 24 hours implying a lack of bacterial growth in this media.

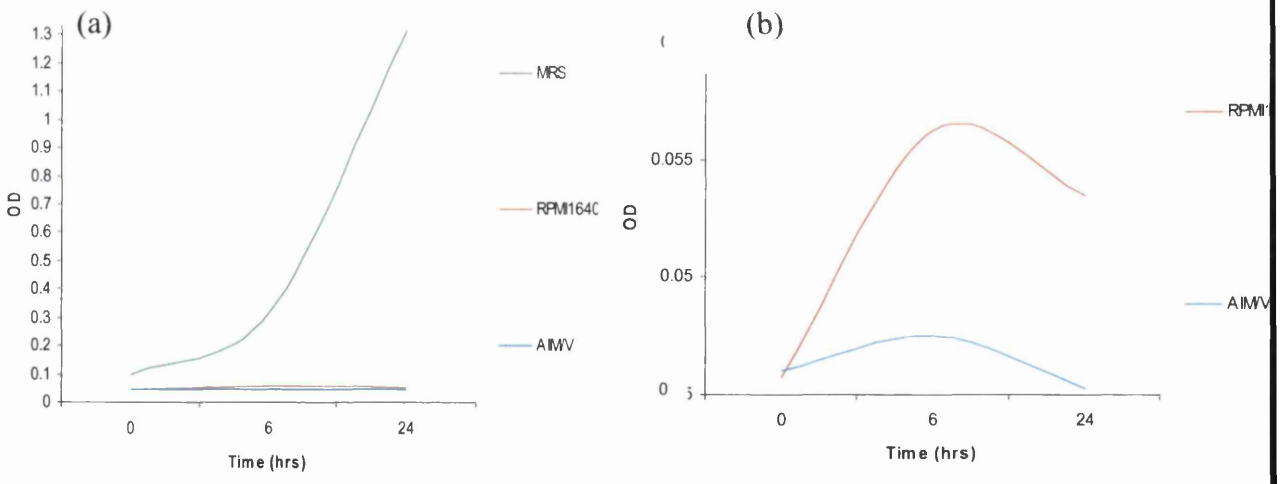


Figure 3.1 Changes in optical density at a wavelength of 600nm (OD) of a liquid culture of the study consortium at starting level of approximately 1×10^6 CFU/ml grown in MRS broth (n=3), complete AIMV (n=3) or complete RPMI (n=3). Comparisons with MRS are provided in (a) and use of different y-axis scale in (b)

allows the differences in growth in complete RPMI versus complete AIMV to be visualised.

Viability of the study consortium in complete RPMI1640 or complete AIMV was then assessed using the conventional microbiological technique of total viable count (TVC). Results of TVC show that bacteria numbers did not increase in complete AIMV, in fact viable bacteria could not be recovered even immediately upon preparing the bacteria in this media at time 0 (Figure 3.2). In compete RPMI there was an initial increase 1×10^6 - 5×10^6 CFU/ml in TVC from 0 – 6 hours but then from 6 – 24 hours numbers declined to approximately the initial starting level (Figure 3.2). In bacterial terms, since the log numbers have not changed these fluctuations in CFUs/ml over the 24 hours reported herein actually represent only a small increase. However the key inference from these results is that the study consortium was stable in complete RPMI1640, a medium commonly used to culture mononuclear cells, but not complete AIMV. Results are consistent with the previous OD measurements. Therefore all further work was undertaken in complete RPMI.

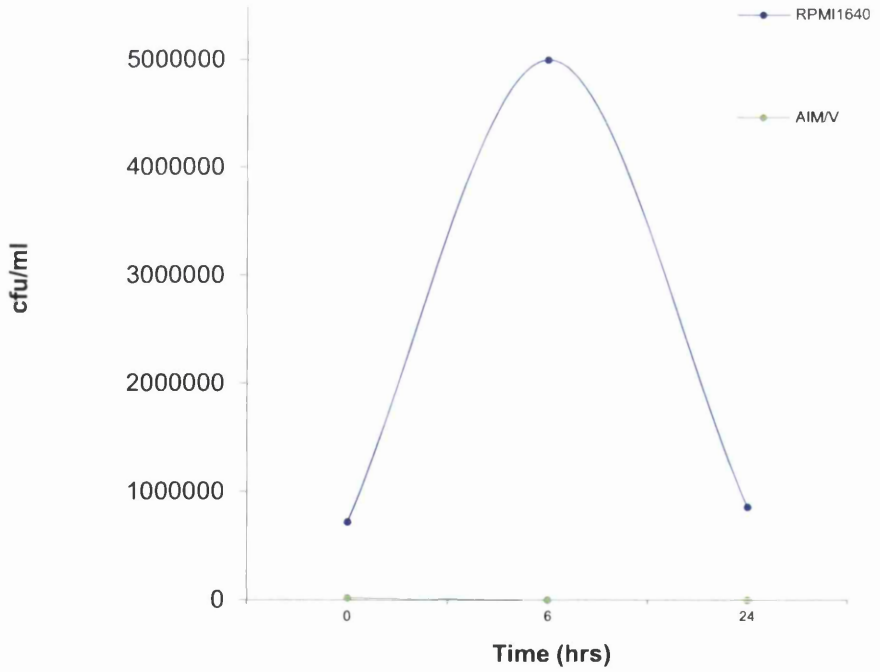


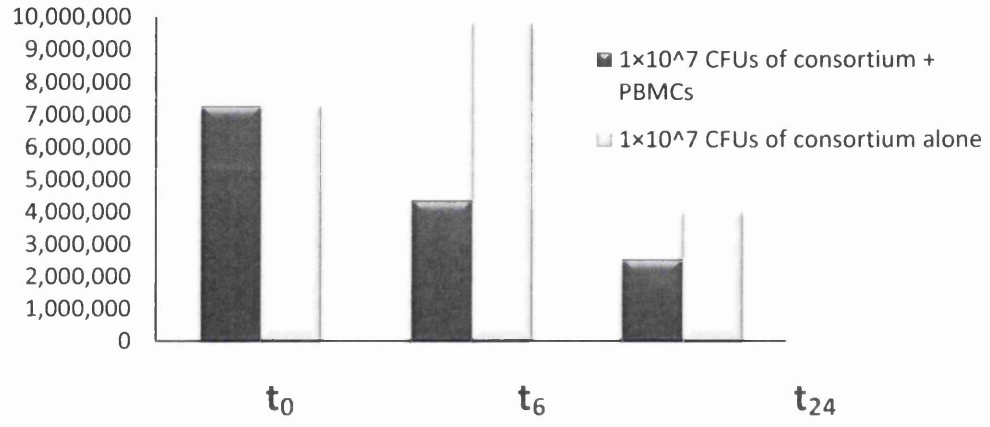
Figure 3.2 Comparison of the growth of the study consortium in complete RPMI or complete AIMV.
Survival of probiotic strains in RPMI1640 or AIM/V culture media was quantified by total viable counts (TVC) on agar plates. Briefly, the contents of a capsule containing 500 mg of the study consortium at 1×10^{10} CFUs was added to each culture medium and then serially diluted down to 1×10^{7} CFU in 4.5 ml of culture medium. At time 0 (T_0), and after 6 (T_6), 24 (T_{24}) and 48 hours (T_{48}), 500µl from the 1×10^{7} CFU dilution was serially diluted in maximum recovery diluent (MRD) to concentrations ranging from 1×10^{6} to 1×10^{2} and added dropwise to MRSx and MRS agar plates. Plates were transferred to an anaerobic incubator for 2-3 days prior to enumeration of colonies via total viable counts.

3.3.2 Optimising culture of bacteria with mammalian cells by assessing the viability of the study consortium in cell culture medium during co-culture with PBMCs

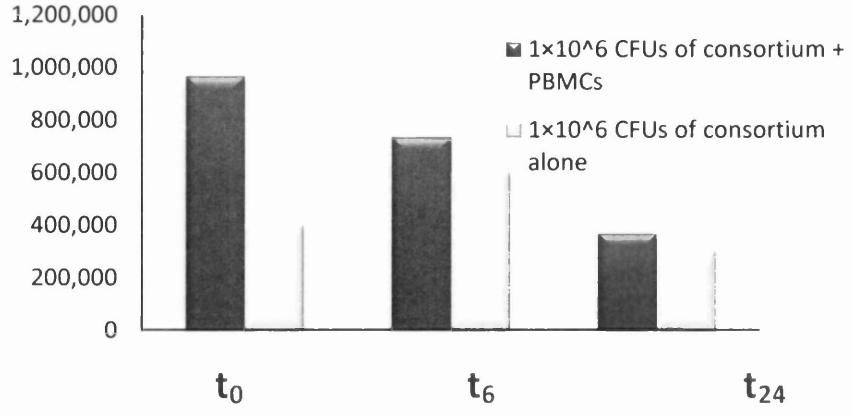
Since the foundation of the *in vitro* model was the co-culture of mononuclear cells with live study consortium bacteria, having determined above that the bacteria remained viable in complete RPMI it was imperative to determine the viability during co-culture with mammalian cells. Different starting concentrations of bacteria (10^7, 10^6 and 10^4 CFUs/ml) were co-cultured with PBMCs at 10^6/ml. Current results indicated that organisms at an initial starting concentration of 1×10^{7} CFU/ml generally proliferated better alone than in co-culture with PBMCs. At T_0 approximately 7.5×10^{6} CFU/ml were present reducing markedly within 6 hours to approximately 4.5×10^{6}. In sharp contrast, growth rates increased when organisms were cultured in the absence of PBMCs reaching 1×10^{7} CFU/ml at the same time point. By 24 hours although there was a general depletion in the number of viable organisms remaining; approximately twice as many viable organisms were present when strains were cultured exclusively in supplemented RPMI1640 compared to when strains were cultured simultaneously with PBMCs; values were approximately 4.0×10^{6} and 2.0×10^{6}, respectively, at 24 hours (Figure 3.3 (a)). At an initial value of 1×10^{6} CFU/ml strains generally proliferated better in the presence of the PBMCs than in the absence with values reaching approximately 9.75×10^{6} and 4.0×10^{6} CFU/ml respectively at T_0 and gradually decreasing at T_6. However, the rate of decrement was greater when strains were cultured in media alone than in the presence of PBMCs approximate values were 7.4×10^{5} and 6.03×10^{5} CFU/ml respectively. By 24 hours the

trend was repeated approximate values were 3.8×10^4 and 3.2×10^4 (Figure 3.3 (b)). Conversely at an initial value of approximately 1×10^4 CFU/ml growth rates were similar regardless of prior culture conditions. At T_0 approximately 1×10^4 CFUs/ml viable strains were recovered alone and in co-culture with PBMCs values increased markedly and doubled within 6hrs to approximately 4.0×10^4 By 24 hours although there was a general depletion in the number of viable strains to just above the initial starting value at approximately 2×10^4 (Figure 3.3 (c)).

(a)



(b)



(c)

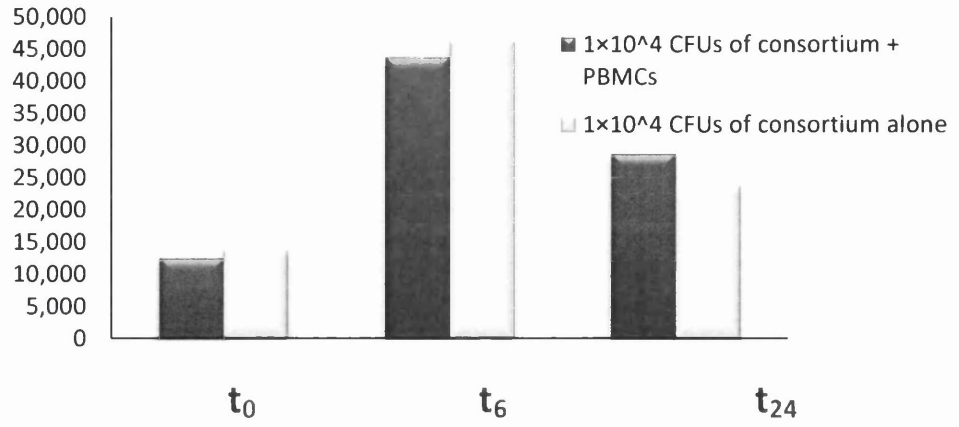


Figure 3.3. Growth rate of a co-culture of the study consortium bacteria in the presence of PBMCs cultured in complete RPMI1640 (± SEM).
Study consortium bacteria at different starting CFUs/ml (a) $1x10^7$, (b) $1x10^6$, and (c) $1x10^4$ were co-cultured with (n=2) or without PBMCs (10^6/ml) over 24 hours and total viable counts of bacteria monitored t_0 (0hr), t_6 (6 hours) and t_{24} (24 hours). Results are an average of two experiments.

3.3.3 Effects of the study consortium on cytokine production from PBMCs and CBMCs *in vitro*.

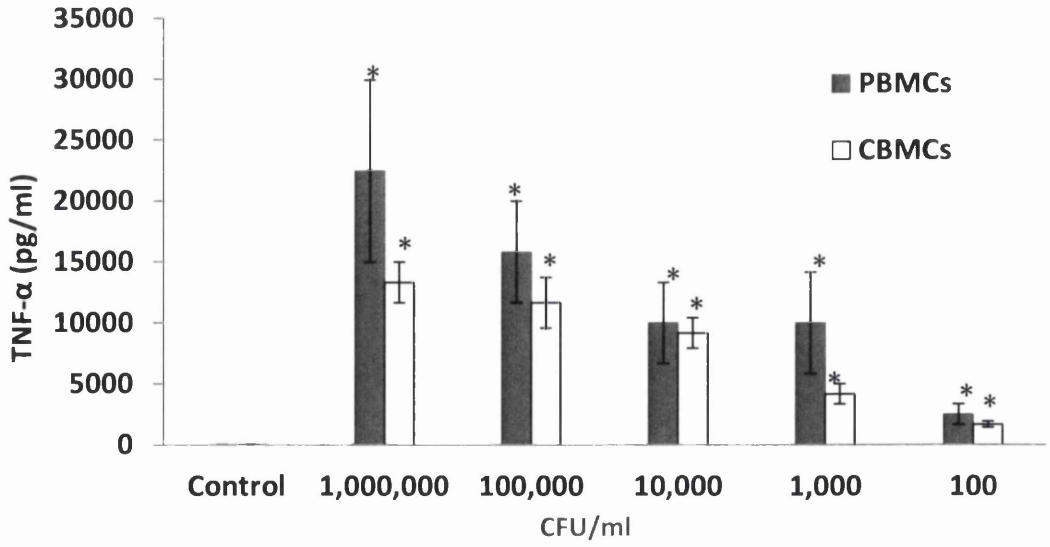
The results presented above set the tone for the experimental conditions required for cytokine analysis. The immunomodulatory effects of the study consortium on cytokine responses from mononuclear cells (PBMCs or CBMCs) co-cultured with a dose course of the consortium over 24hr was determined. Mononuclear cells cultured alone served as an internal experimental control. The cytokines measured where TNF-α, IL-12p70, IL-10, IL-6 and IL-1β (Figure 3.4a – f). Generally PBMCs or CBMCs cultured alone (control) generated negligible baseline cytokine production. Conversely, mononuclear cells from both groups responded to the consortium in a dose dependent manner. Other than at the lowest dose of 10^2 CFUs/ml for IL-1β, IL-6 and IL-12p70 the study consortium induced a statistically significant increase in all the studied cytokines for both CBMCs and PBMCs when compared to the untreated controls. The effect of the consortium on TGFβ1 was also considered. Since TGFβ1 typically occurs in a latent form not readily detectable by ELISA, cell/bacteria free supernatants were also acidified then neutralised to release active TGFβ1. The capacity of the consortium to induce TGF-β1 responses after activation by CBMCs and PBMCs occurred in a dose independent manner. These responses when compared to the controls where not statistically significant at any dose. Interestingly the difference in TGF-β1 production between PBMCs and CBMCs measured after activation followed a similar trend as all the other cytokines measured with PBMC

again upregulating stronger TGF-β1 immune responses to the presence of the consortium compared to CBMCs, these difference were however only statistically significant at low consortium doses of 10^3 ($P < 0.019$) and 10^2 ($P < 0.048$) CFUs/ml.

The cytokine response made by adults versus neonates was also compared and although there were no statistical differences in TNF-α, IL-6 and IL-1β production between PBMCs and CBMCs in response to the study consortium, IL-12p70 and IL-10 production was significantly impaired in the neonate compared to the adult (Table 3.2.).

The results presented herein serve to highlight the distinct developmental differences between neonatal and adult immunity as represented by differences in cytokine kinetics between PBMCs and CBMCs co-cultured in the presence of the consortium. Generally CBMCs demonstrated an attenuated ability to induce a cytokine response in response to co-culture with the consortium at the strongest doses in comparison to adult PBMCs. PBMCs always produced the strongest cytokine responses compared to CBMCs at the highest consortium dose for all cytokines measured. The most striking difference was the reduced capacity for IL-12p70 production by CBMCs in response to the consortium (Figure 3.4b). Overall the ability of the consortium to induce cytokines responses in CBMCs versus PBMCs samples varied with each of the cytokines selected for analysis.

(a)



(b)

*

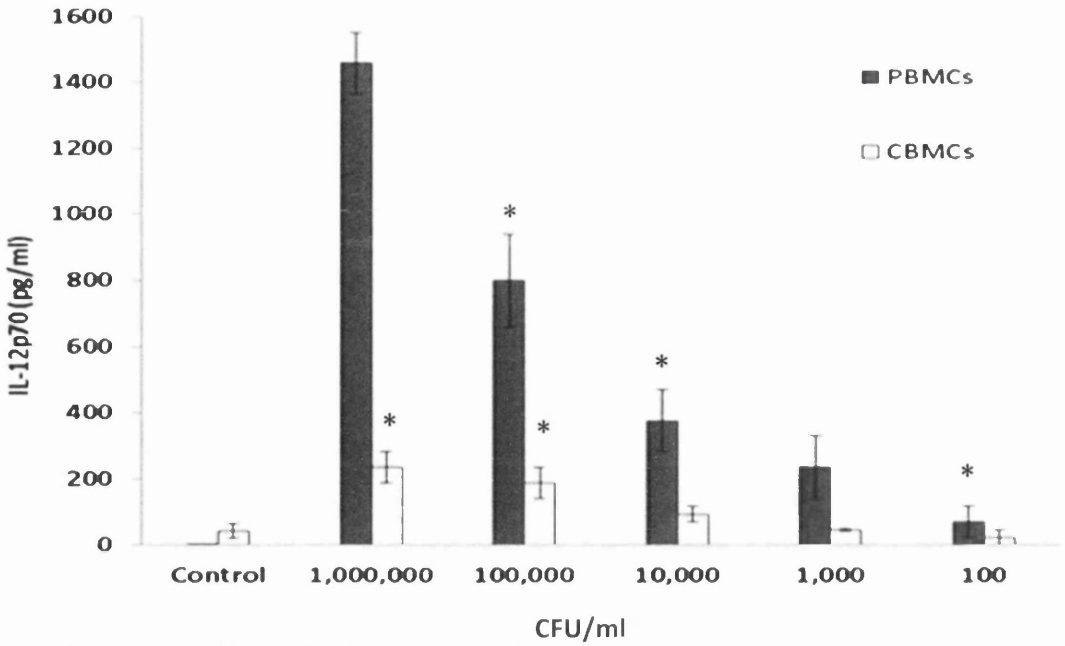
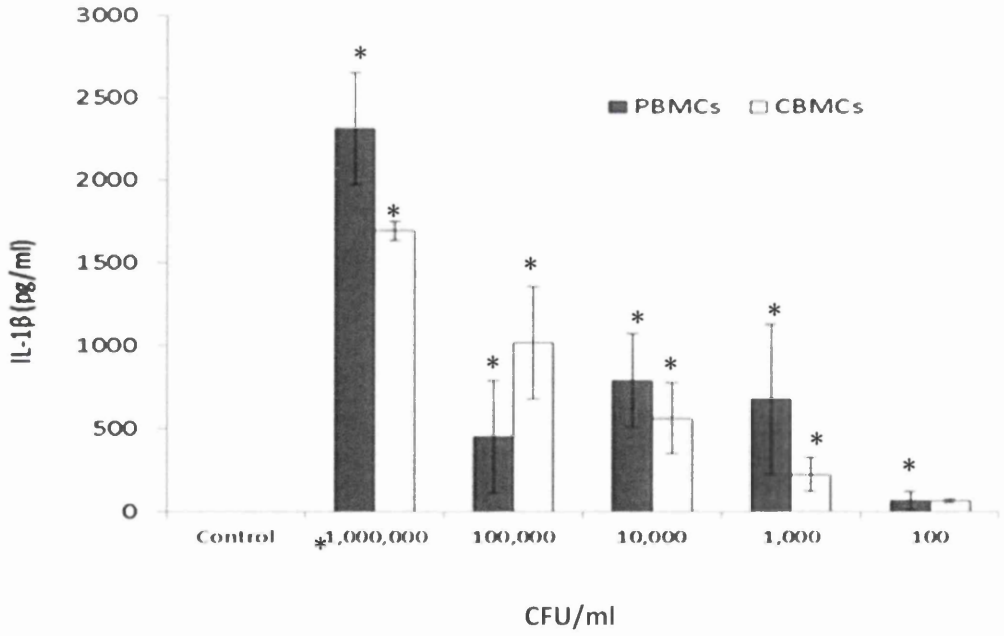


Figure 3.4 (i) The effect of a dose course of a study consortium of probiotics on cytokine production in pg/ml (± SEM) by n = 25 adult peripheral blood mononuclear cells (PBMCs dark grey bars) and n = 25 umbilical cord blood mononuclear cells (CBMCs white bars) in comparison to the unstimulated control. P-values < 0.05 are denoted on the graphs with an. * PBMCs or CBMCs at 10^6/ml were co-cultured with doses of the study consortium (ranging from 106 to 102 CFUs/ml indicated on the x-axis) for 24hr. Cell/bacteria free culture supernatants were harvested for later analysis of (a) TNF-α and (b) IL-12p70 using specific ELISAs in accordance with the manufacturer's instructions.

(c)

(d)



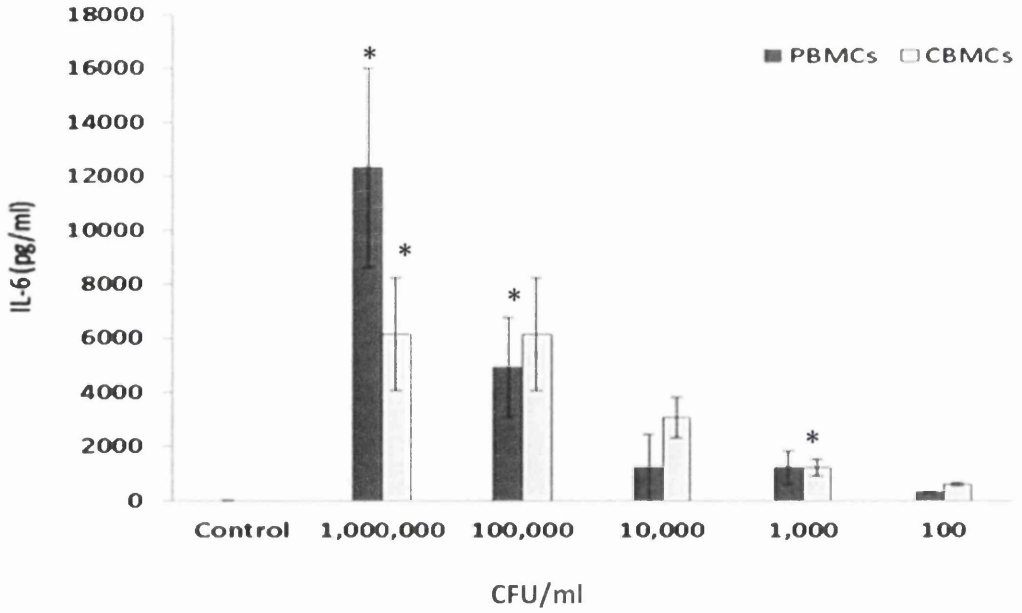
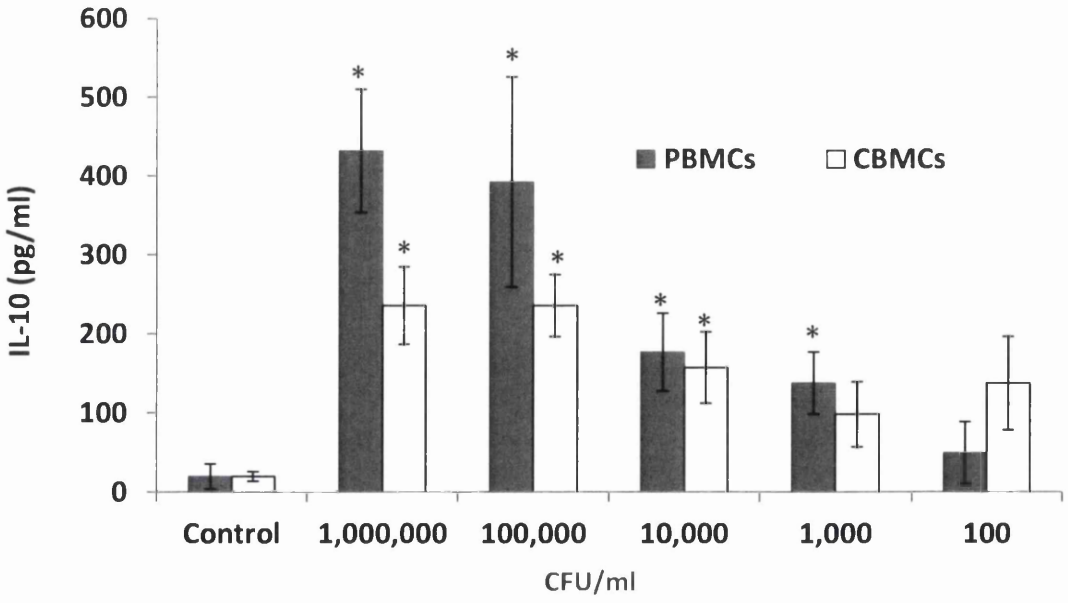


Figure 3.4 (ii) The effect of a dose course of a study consortium of probiotics on cytokine production in pg/ml (± SEM) by n = 25 adult peripheral blood mononuclear cells (PBMCs dark grey bars) and n = 25 umbilical cord blood mononuclear cells (CBMCs white bars) in comparison to the unstimulated control. P-values < 0.05 are denoted on the graphs with an. * PBMCs or CBMCs at 10^6/ml were co-cultured with doses of the study consortium (ranging from 10^6 to 10^2 CFUs/ml indicated on the x-axis) for 24hr. Cell/bacteria free culture supernatants were harvested for later analysis of (c) IL-1β and (d) IL-6 using specific ELISAs in accordance with the manufacturer's instructions.

(e)



(f)

Figure 3.4 (iii) The effect of a dose course of a study consortium of probiotics on cytokine production in pg/ml (± SEM) by n = 25 adult peripheral blood mononuclear cells (PBMCs dark grey bars) and n = 25 umbilical cord blood mononuclear cells (CBMCs white bars) in comparison to the unstimulated control. P-values < 0.05 are denoted on the graphs with an. * PBMCs or CBMCs at 10^6/ml were co-cultured with doses of the study consortium (ranging from 10^6 to 10^2 CFUs/ml indicated on the x-axis) for 24hr. Cell/bacteria free culture supernatants were harvested for later analysis of (e) IL-10 and (f) TGF-β1 using specific ELISAs in accordance with the manufacturer's instructions.

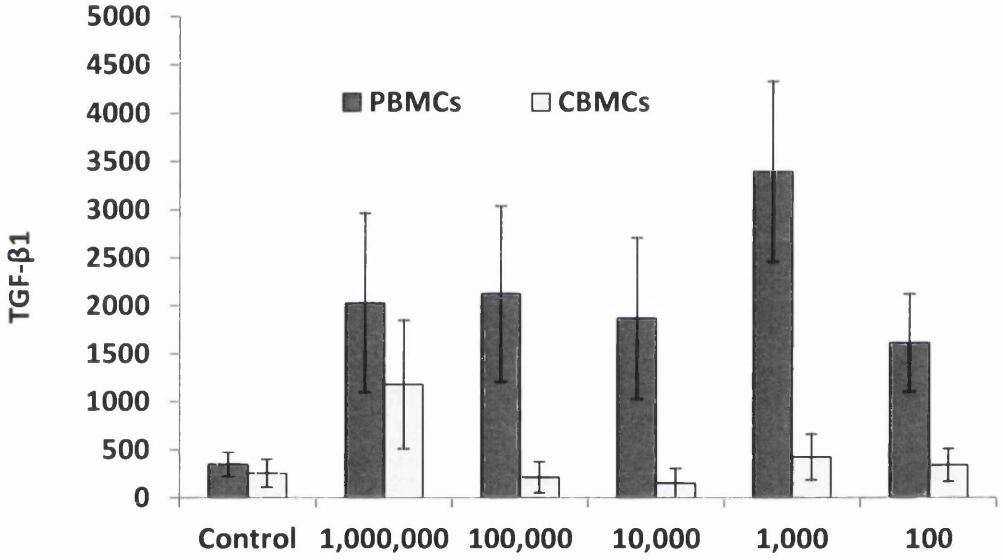


Table 3.2. Results of statistical analysis of effect of each dose (CFUs/ml) of the study consortium on production of IL-12p70 and IL-10 by CBMCs compared with PBMCs. P > 0.05 was considered statistically significant.

Comparisons between PBMCs and CBMCs cytokine responses at each dose	IL-12p70	IL-10	TGF-β1
10^6	0.001	0.001	No significance
10^5	0.002	0.088	No significance
10^4	0.022	0.057	No significance
10^3	0.015	0.006	0.019

10^2	No significance	0.057	0.048

Interestingly statistical significance was not reached at any dose when the effect of study consortium on production of other classical pro-inflammatory cytokines such as TNF-α IL-1β and IL-6 was compared between CBMCs and PBMCs.

3.3.4 Effects of the study consortium on cytokine production from mitogen- and superantigen-stimulated CBMCs and PBMCs.

The study consortium was co-cultured in the presence of CBMCs or PBMCs for 24 hour followed by stimulation with phytohaemagluttinin (PHA) or staphylococcal enterotoxin B (SEB) for a further 24 hour. The dose course experiments conducted above indicated that 1×10^6 CFUs/ml stimulated a significant cytokine response so only this dose was used for these experiments. By culturing bacteria at this dose with MNCs at 1 x 10^6/ml a 1:1 ratio of MNC to consortium was achieved. For all figures/tables the presence of consortium bacteria is represented with (+).

In figure 3.7 it can be seen that SEB and PHA alone induced significant increases in IL-13, IFN-γ and IL-17 by CBMCs and PBMCs. In the presence of the study consortium, the IL-13 response to both SEB and PHA was significantly decreased in PBMCs (P = 0.011 and 0.007) respectively but not by CBMCs. The converse was seen for IFN-γ, with SEB- and PHA-induced IFN-γ responses potentiated in the presence of study consortium by PBMCs (P = 0.036 and 0.018), respectively. This trend was also observed for SEB induced IFNγ by CBMCs (P = 0.017) but not PHA induced IFN-γ production by CBMCs. Notably, in the absence of any stimuli the

study consortium induced IL-13 by CBMCs but IFN-γ by PBMCs. PHA and SEB induced significant increases in IL-17 from PBMCs (P = 0.0007 and 0.00001) respectively but not for CBMCs. Interestingly, the study consortium significantly decreased SEB-induced IL-17 by PBMCs (p=0.003). In marked contrast the presence of the consortium appeared to potentiate PHA- and SEB- induced IL-17 by CBMCs however this did not reach statistical significance (p = 0.099 and 0.066 respectively). The SEB (P=0.026) and PHA (p=0.032) induced IFN-γ responses in the presence of the study consortium were significantly greater by PBMCs than CBMCs. The SEB (P=0.034) induced IL-17 response in the presence of the consortium was also significantly greater by PBMCs than CBMCs.

The current results also indicated that the consortium was able to significantly induce IL-17 (P=0.03), IFN-γ (P=0.02), but not IL-13 (P=0.704), compared to the untreated control, independent of T-cell mitogen stimulation (provided by SEB) or lymphocyte mitogen stimulation (provided by PHA) suggesting that these cytokines can also be induced by the consortium from a non-lymphocyte source. These data indirectly correlate with the results showing that CD14 and CD14 negative mononuclear cells fractions are able to respond to the consortium via cytokine production (See 3.3.6).

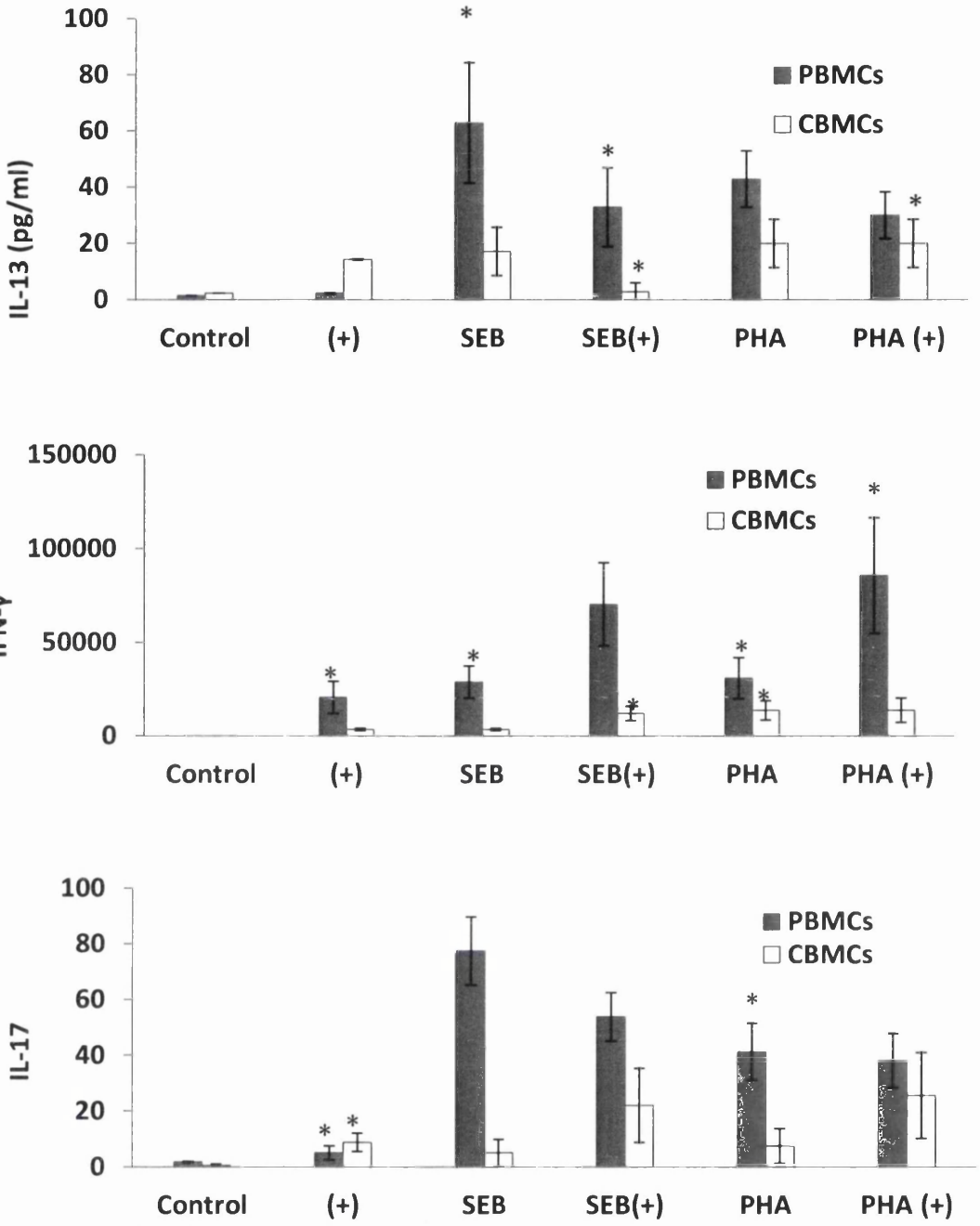


Figure 3.5 Effects of consortium on SEB and PHA induced cytokine production
Employing n = 15 umbilical cord blood mononuclear cells (white bars) and n = 15 adult peripheral blood mononuclear cells (grey bars). Mononuclear cells from umbilical cord and adult peripheral blood were cultured for 24 hours in the presence of 10^6 CFUs/ml of the consortium denoted by (+) and then a further 24 hours in the presence of SEB (200ng/ml) or PHA (1μg/ml). Cell-free culture supernatants were prepared and then (a) IL-13, (b) IFN-γ, and (c) IL-17 (pg/ml ± SEM) measured using specific ELISAs. The student t test was employed to determine whether there was a statistically significant difference in mononuclear cell response between (1) consortium and the control. (2) SEB or PHA stimulated mononuclear cells versus the control and (3) SEB or PHA stimulated mononuclear cells alone versus SEB or PHA stimulated mononuclear cells in the presence of the consortium. Details of p-values < 0.05 are denoted on the graphs with an *

3.3.4.1 Effects of the study consortium on IFNγ/IL-13 ratio.

The PHA and SEB induced IFN-γ/IL-13 ratio (Fig 3.6) was significantly enhanced in the presence of the study consortium by both PBMCs and CBMCs, highlighting the potential of the consortium to influence Th1/Th2 polarisation in favour of Th1 responses. This polarizing effect is consistent with emerging research by (Ghadimi *et al.* 2010) indicating that LAB enhance autophagic ability of mononuclear phagocytes by increasing Th1 autophagy-promoting cytokine IFN-γ levels and reducing Th2 autophagy-restraining cytokines IL-4 and IL-13. Regulation of the onset and/progression of potential IgE-mediated allergy responses requires an appropriate ratio of Th1/Th2/Th17 cytokines to exert control over physiologically, and immunologically regulated processes involved during the onset and progression of allergy. The study consortium has been able to induce both Th1 promoting cytokines, such as IFN-γ, and immunosuppressive Th2 cytokines like IL-13.

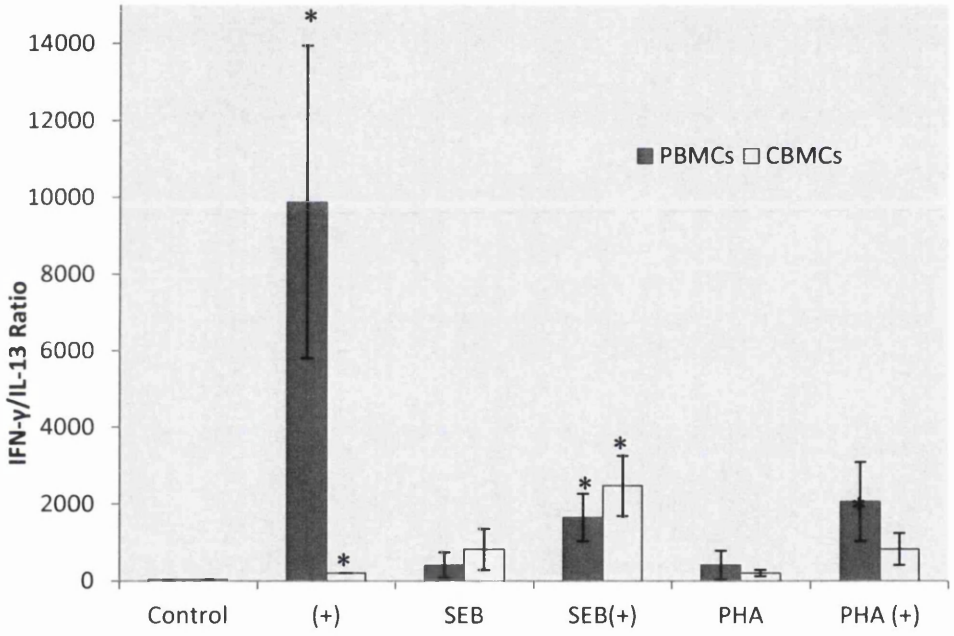


Figure 3.6 Ratio of IFNγ/IL-13 levels in supernatants
CBMCs (n = 15) or PBMCs (n = 15) were cultured for 24 hours in the presence of 10^6 CFUs/ml of the consortium denoted by (+) and then a further 24 hours in the presence of SEB (200ng/ml) or PHA (1μg/ml). Cell free culture supernatants were prepared and then IL-13 and IFN-γ measured prior to calculation of the ratio of these two cytokines. The student t test was employed to determine whether there was a statistically significant difference in mononuclear cell response between (1) consortium and the control. (2) SEB or PHA stimulated mononuclear cells versus the control and (3) SEB or PHA stimulated mononuclear cells alone versus SEB or PHA stimulated mononuclear cells in the presence of the consortium. Details of p-values < 0.05 are denoted on the graphs with an *

3.3.4.2 Effects of the study consortium on IL-17/IL-13 ratio

To further investigate the potential role of the consortium upon novel pro-inflammatory cytokines also involved in the pathogenesis of IgE mediated allergy, the IL-17/IL-13 ratio induced by the consortium was also monitored (Fig 3.7). IL-17, a novel pro-inflammatory Th1 cytokine produced by activated T helper cells (Aarvak *et al.* 1999), plays an upstream role in T cell-triggered inflammation and haematopoiesis by stimulating stromal cells to secrete other cytokines and growth factors. IL-17 is capable of stimulating production of the pro-inflammatory cytokine IL-6 (also analysed in this study), proliferation of T cells as well as growth and differentiation of $CD34^+$ human progenitors into neutrophils (Fossiez *et al.* 1996; Onishi and Gaffen 2010). Current results indicated that the IL-17/IL-13 ratio was not significantly altered by the consortium in both PBMCs and CBMCs under any of the treatment conditions, and there were no significant difference in the ratio between PBMCs and CBMCs. Although the IL-17/IL-13 ratio appeared to increase when CBMCs where exclusively treated with SEB or when SEB stimulated PBMCs were treated with the consortium, neither of these conditions reached statistical significance ($P=0.06$ and 0.064 respectively. However this could be attributed to the small sample size employed for analysis.

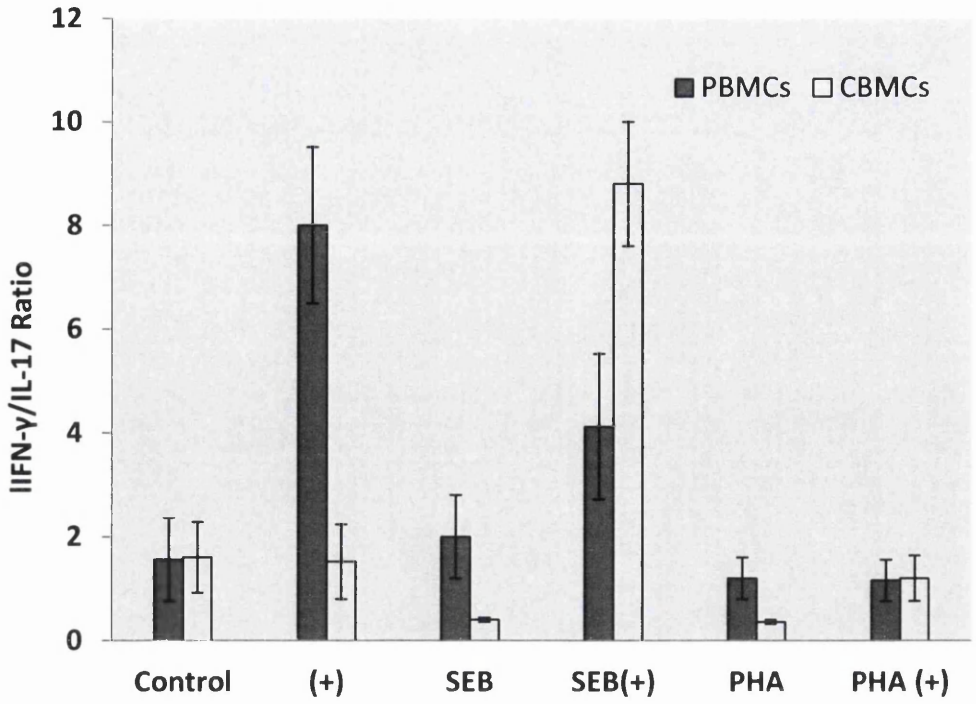


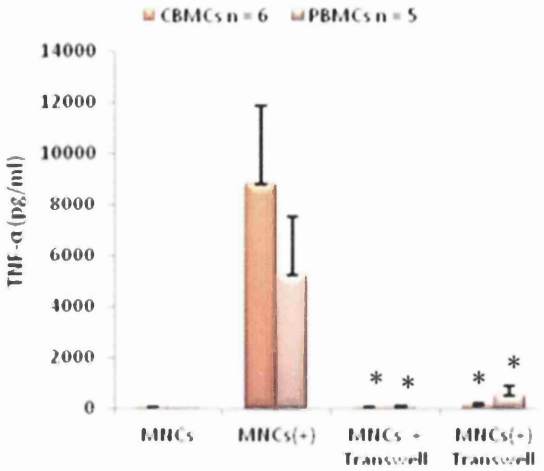
Figure 3.7 Ratio of IL-17/IL-13 levels in supernatants
CBMCs (n = 15) or PBMCs (n = 15) were cultured for 24 hours in the presence of 10^6 CFUs/ml of the consortium denoted by (+) and then a further 24 hours in the presence of SEB (200ng/ml) or PHA (1μg/ml). Cell free culture supernatants were prepared and then IL-17 and IL-13 measured prior to calculation of the ratio of these two cytokines. The student t test was employed to determine whether there was a statistically significant difference in mononuclear cell response between (1) consortium and the control. (2) SEB or PHA stimulated mononuclear cells versus the control and (3) SEB or PHA stimulated mononuclear cells alone versus SEB or PHA stimulated mononuclear cells in the presence of the consortium. Details of p-values < 0.05 are denoted on the graphs with an *

3.3.5 Requirement for contact between study consortium bacteria and mononuclear cells.

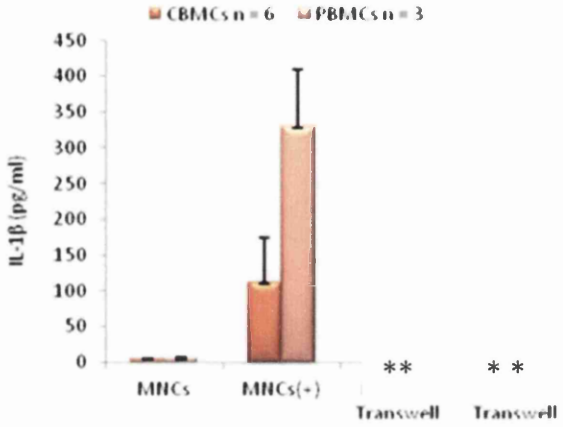
The effect of study consortium bacteria on MNCs could reflect secreted bacterial products or require direct contact between the bacteria and the mammalian cells. This was investigated using transwells with a pore size of 0.4μm to prohibit contact between the bacteria and the MNCs. The presence of a transwell separating mononuclear cells and study consortium into distinct compartments abolished the cytokine-inducing effect of the bacteria (Figure 3.9). This was seen for all the cytokines included for analysis: TNF-α (Figure 3.9(a)), IL-10 (Figure 3.9 (b)) and IL-1β (Figure 3.9 (c)). An additional dose response curve of the study consortium was

performed with IL-12p70 as the output , and this confirmed that contact was required between bacteria and PBMCs or CBMCs at all doses of bacteria studied (Figure 3.10).

(a)



(b)



(c)

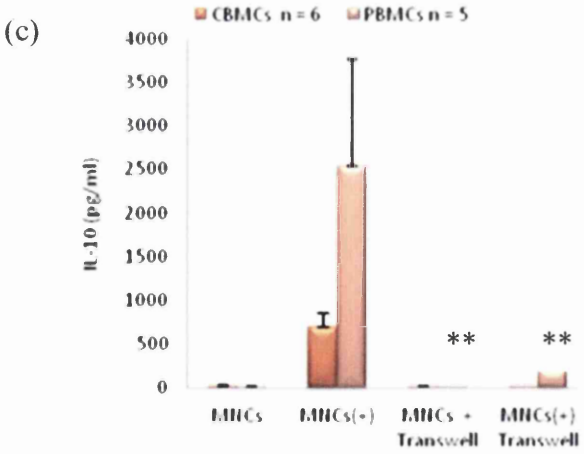


Figure 3.8. Requirement for contact between study consortium and mononuclear cells for cytokine responses.
Mononuclear cells from umbilical cord (CBMCs) and adult peripheral blood (PBMCs) were cultured for 24 hours in the presence of 10^6 CFUs of the consortium and remained in contact or were separated by a 0.4μm transwell. Cell free culture supernatants were prepared and (a) TNF-α, (b) IL-1β (c) IL-10, measured using specific ELISAs. Significant differences ($p < 0.05$) between the response by CBMCs and PBMCs in response to the consortium in the presence and the absence of a transwell are denoted by *

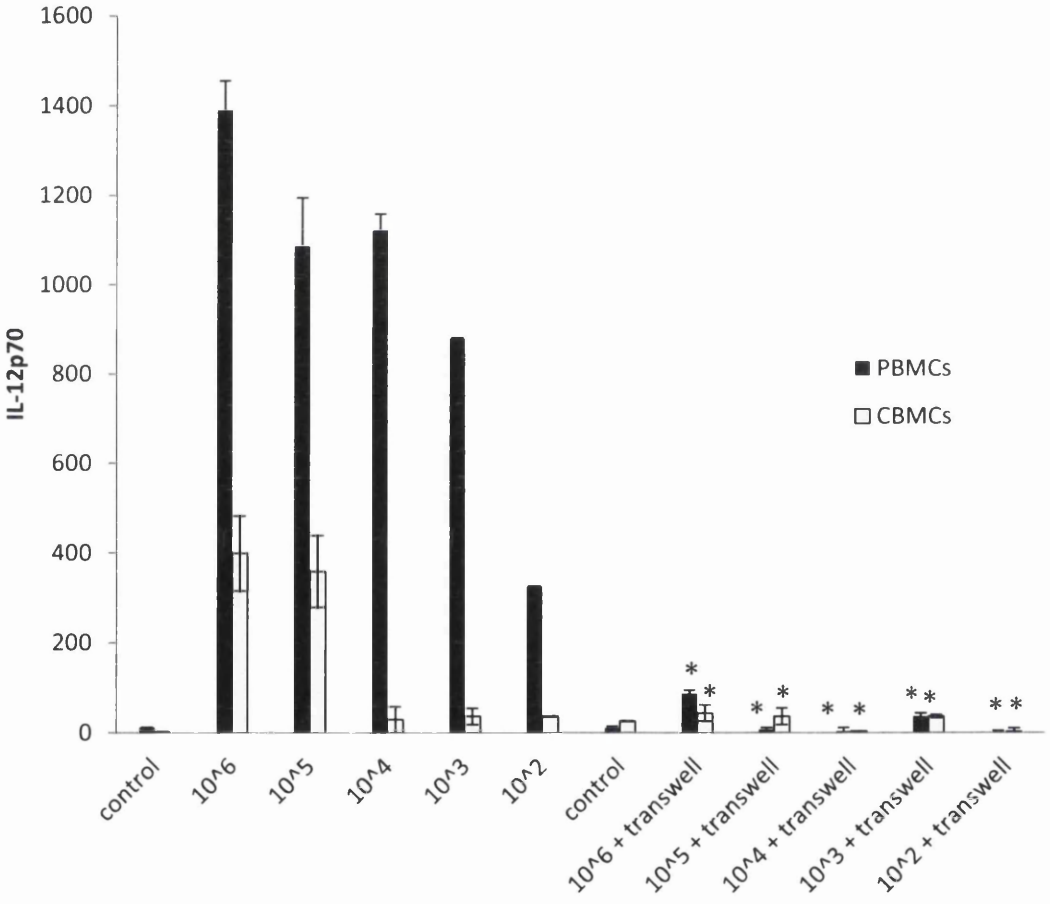


Figure 3.9 Requirement for contact between study consortium and mononuclear cells for IL-12p70 response.
Mononuclear cells from umbilical cord (n = 3) and adult peripheral blood (n = 3) were cultured for 24 hours in the presence of different doses of the study consortium and were allowed to remain in contact or were separated by a 0.4μm transwell. Cell free culture supernatants were prepared and then IL-12p70 measured using specific ELISAs.

Significant differences ($p < 0.05$) between the response by CBMCs and PBMCs in response to the consortium in the presence and the absence of a transwell are denoted by *

3.3.6 $CD14^+$ monocytes are required for the response by MNCs to the study consortium

Having confirmed the requirement for contact between MNCs and study consortium bacteria, a candidate group of responsive cells within the MNC preparation were studied. Monocytes were purified from total MNC preparations by positive selection with anti-CD14 magnetic microbeads. MNCs were first isolated from adult peripheral blood or umbilical cord blood samples by density gradient centrifugation over Histopaque®-1077 as in (2.1.3). Contaminating glycophorin positive red blood cells (RBCs) were first removed from the mononuclear cell preparation by labelling MNCs with glycophorin magnetic microbeads (Miltenyi Biotec), in accordance with the manufacturer's guidelines. Labelled MNCs were then applied to the autoMACS and programme 'depleteS' used to deplete the MNC preparations of RBCs. RBC-depleted MNCs eluted from the magnetic column were then used for preparation of $CD14^+$ monocytes and $CD14^-$ depleted MNCs. MNCs were counted and then labelled with CD14 magnetic microbeads. The $CD14^-$ depleted fraction was used as a source of MNCs virtually devoid of monocytes so that comparison of glycophorin depleted MNCs, $CD14^+$ monocytes and $CD14^-$ depleted MNCs could be made. All preparations were monitored using flow cytometry with anti-CD14 antibody (Figure 3.10).

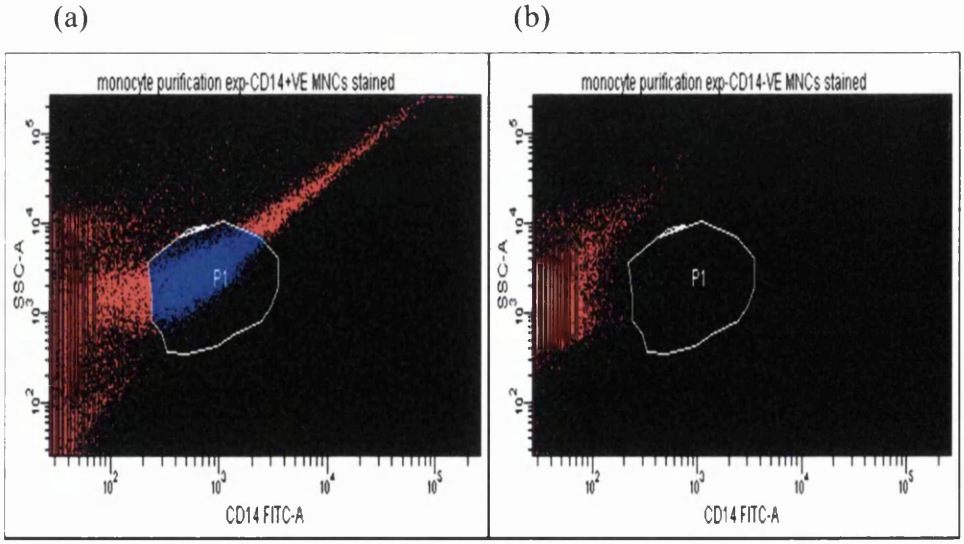


Figure 3.10 Flow cytometry was used to monitor preparation of $CD14^+$ monocytes. The relative abundance of $CD14^+$ cells in (a) positively selected $CD14^+$ monocytes, and (b) $CD14^-$ depleted MNCs is shown. Reactivity to anti-human CD14: FITC antibody is shown on the x-axis and SSC was used as the y-axis parameter. Total MNCs, $CD14^+$ monocytes and $CD14^-$ depleted MNCs (all at 10^6/ml) were exposed to 10^6 CFUs/ml of the

study consortium for 24hr. TNF-α was measured in the cell/bacteria free culture supernatants.

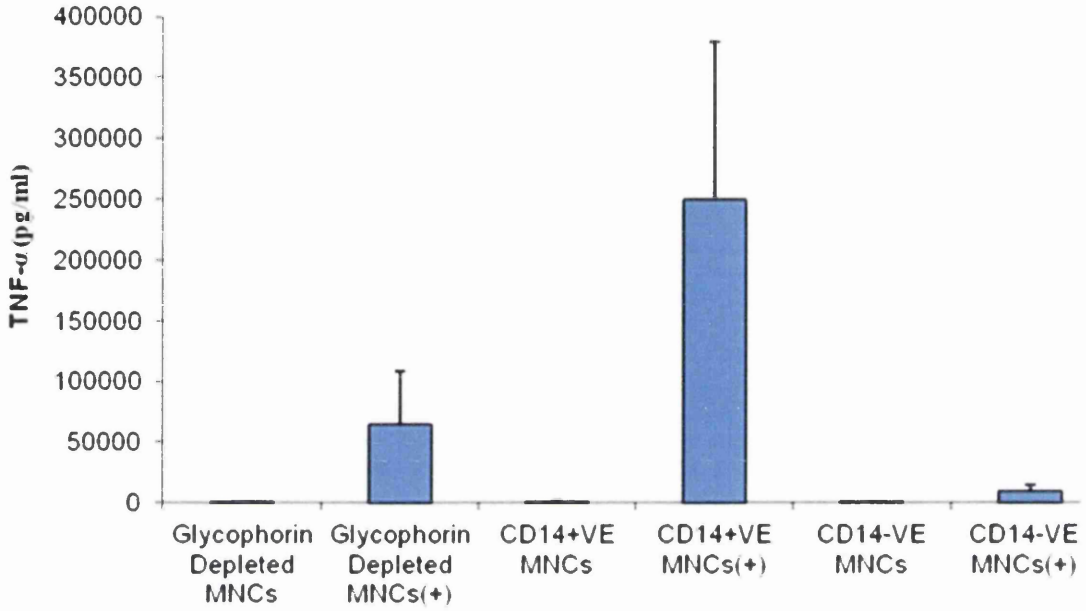


Figure 3.11 Contribution of CD14$^+$ monocytes to cytokine response to study consortium bacteria.
A single dose of study consortium bacteria (10^6 CFUs/ml) were co-cultured with glycophorin depleted MNCs (n=3), CD14$^+$ monocytes (n=3) or CD14$^-$ depleted MNCs (n=3) (10^6 cells/ml) for 24 hours with later analysis of TNF-α.

3.4 Discussion

This *in vitro* study analysed the immunomodulatory attributes of a study consortium of probiotic strains hypothesised to impact positively on immune development and impart anti-allergic immune function. These immunostimulatory properties were assessed by testing interactions of the consortium with mononuclear cells prepared from human adult peripheral blood and umbilical cord blood, and responses were measured in terms of cytokine production by mononuclear cells in our *in vitro* model system.

Due to the need to co-culture bacteria with mononuclear cells in this model system it was imperative to establish the effects of standard mammalian cell culture systems on the study consortium viability. The present results indicate that the study consortium maintained viability in cell culture media RPMI1640/Glutamax supplemented with 5% FBS and 2-ME for at least 24 hours, in comparison to growth rates in the serum free medium AIMV media, as indicated by the total viable counts. Comparable results were obtained using optical density measurements where turbidity was applied as a proxy for bacterial proliferation. Bacteria viability was retained in the presence of mononuclear cells in RPMI/FBS/2-ME so this media was used for the remainder of the *in vitro* experiments. Although these steps ensured robustness of the study, research has demonstrated that viability of probiotic bacteria is not an absolute pre-requisite for cytokine induction in response to interaction with haematopoietic cells, with heat killed probiotic bacteria also displaying immunogenicity (Sashihara *et al.* 2006).

3.4.1 Immunomodulatory effect of the study consortium on production of inflammatory cytokines by umbilical cord blood and adult peripheral blood mononuclear cells

In previous studies, lactobacilli and bifidobacteria strains have been demonstrated to have a strong cytokine inductive capacity on PBMCs *in vitro*. IFN-γ, TNF-α, IL-1β, IL-6 , IL-12 and IL-10 have all been induced experimentally upon interaction with probiotic bacterial strains (Miettinen 1998; Haller 2000; Lammers *et al.* 2003; Niers 2005; Shida 2006; Helwig *et al.* 2006; Foligne *et al.* 2007; Drouault-Holowacz *et al.* 2006). Similarly, the current study demonstrated a similar capacity for consortium strains to induce TNF-α, IL-12p70, IL-10, IL-6 and IL-1β in a dose dependent fashion by CBMCs and PBMCs. The most notable differences were the dramatically reduced response by CBMCs for production of IL-12p70 at all doses and IL-10 at high doses of the study consortium. This may in part be reflective of the immature developmental stage of newborn immunity (Nilsson 2004; Gabrielssons 2001). However, interpretation must take into account that kinetics of TNF-α, IL-6 and IL-1β production were comparable between adults and neonates. Although at the highest consortium dose production of these 3 key pro-inflammatory cytokines was attenuated in CBMCs. These observations are in line with numerous studies

indicating that newborns are deficient in their ability to produce the important Th1 cytokine IL-12p70 (Nilsson *et al.* 2004; Tulic *et al.* 2011) a predilection that supports the framework for atopic sensitisation in infancy in genetically predisposed individuals (Upham *et al.* 2002; Prescott *et al.* 2003; Holt *et al.* 2005). Additionally post-natal vulnerability to mount stronger Th2 immune responses concomitant with weaker allergen-specific Th1 response and variation in antigen presenting cell function in early life supports the postulate of the intrinsically Th2 skewed nature of newborn immunity. Fortunately, this scenario improves post-natally upon immunological maturity, shaped by the quality of immune education received *ex utero;* of which, interactions with immunomodulatory probiotic strains of the microbiota is crucial. This is one of few studies considering the impact of probiotics on cytokine production by umbilical cord blood mononuclear cells *in vitro* and provides an insight into how newborn immunity might be shaped at an immunological level according to early interactions with probiotic bacteria.

3.4.2 Immunomodulatory effect of the study consortium on production of anti-inflammatory cytokines by umbilical cord blood and adult peripheral blood mononuclear cells

This *in vitro* studied demonstrated that the consortium was directly capable of inducing the anti-inflammatory cytokine IL-10 without the need of additional mononuclear cell mitogenic stimulation and was in line with data from a clinical study demonstrating that ingestion of *Lactobacillus rhamnosus* is associated with high serum levels of IL-10, which is preceded by a rise in mitogen-induced IL-10 from PBMCs (Pessi *et al.* 2000). Current results are also in keeping with a study demonstrating that *in vitro* addition of probiotics to PBMC cultures resulted in enhanced proliferation and production of IFN-γ, IL-10, and TNF-α (Flinterman *et al.* 2007).

The effect of the study consortium on another anti-inflammatory cytokine TGF-β1 from CBMCs and PBMCs was also considered. Since TGF-β1 typically occurs in a latent form not readily detectable in most ELISAs utilising monoclonal antibodies, samples were acidified to release detectable TGF-β1. Levels of TGF-β1 detectable in culture supernatants were higher for PBMCs than CBMCs. The study consortium potentiated TGF-β1 levels in PBMCs but not CBMCs. The ability of another

probiotic strain, *Lactobacillus johnsonii,* to induce TGF-β1 has been demonstrated using the intestinal epithelial cells line Caco-2 and PBMCs (Haller *et al.* 2000).

Results of the current study supports observations that the anti-inflammatory IL-10 and TGF-β1 mediated responses can be induced by probiotic bacteria. For instance *Lactobacillus paracasei* a constituent of the current study consortium induced the development of a population of $CD4^+$ T lymphocytes with low proliferative capacity and high TGF-*β* and IL-10 production, reminiscent of Tr1 cells implicated in oral tolerance and gut homeostasis (von der Weid *et al.* 2001). Since it is known that probiotics stimulate dendritic cells to enhance IL-10 production (Drakes *et al.* 2004) and that IL-10 is essential for Tr1 expansion (Wakkach *et al.* 2003), probiotics may induce the development of Tr1 through dendritic cells stimulation. Since IL-10 and TGF-β are the archetypal cytokines involved in differentiation of Treg populations the effects of perinatal supplementation in enhancing peripheral blood levels of putative Tregs was considered in chapter 6.

3.4.3 Effects of the consortium on PHA and SEB induced responses

This part of the study considered the immunomodulatory impact of the study consortium on production of the prototypic Th1 (IFN-γ), Th2 (IL-13) and Th17 (IL-17) cytokines. These responses were first monitored by separately priming CBMCs and PBMCs with either (PHA) or (SEB) alone; or separately priming CBMCs and PBMCs with either (PHA and IFN-γ) or (SEB and IFN-γ).

Interestingly, the study consortium potentiated IFN-γ responses by both SEB and PHA stimulated PBMCs but only SEB stimulated CBMCs. The opposite effect was seen for IL-13 production. PHA and SEB were both able to significantly stimulate IL-13 production by CBMCs and PBMCs but this response was attenuated in the presence of the study consortium for both PHA and SEB for PBMCs but, only SEB for CBMCs. Overall; these results indicate that the study consortium favours Th1 responses to the detriment of a Th2 response. This is consistent with previous studies indicating that lactobacilli and bifidobacteria indicating increased *in vitro* production of Th1 cytokines from probiotic treated PBMCs or CBMCs (Cross *et al.* 2001) Moreover NK cell IFN-γ production has also been demonstrated to increase in response to probiotics (Ogawa *et al.* 2006).

Generally the IFN-γ response by CBMCs was significantly reduced when compared to PBMCs under all treatment conditions, an observation that supports the attenuated CBMC IL-12p70 response was compared to the PBMC IL-12p70 in response to the consortium. The current data is in keeping with all published studies highlighting immunological difference between adults and newborns in inducing Th1 immunity in response to stimuli by micro-organisms (White *et al.* 2002).

IL-12p70 is a key cytokine in the initiation of IFN-γ production by human PBMCs, as well as monocytes (Bekeredjian-Ding *et al.* 2006) . Given the synergy between IL-12p70 and IFN-γ in maintaining Th1 responses, the previously observed lack of IL-12p70 production by CBMCs, in response to the study consortium in part, explains the lack of IFN-γ produced by activated CBMCs. Induction of IL-12p70 in response to the consortium is important for subsequent generation of IFN-γ and potentiating Th1 immune responses (Hessle *et al.* 1999; Kato *et al.* 1999). Current results were in line with *in vitro* experiments by other authors, demonstrating that the stimulation of PBMCs from healthy donors with lactobacilli and bifidobacteria species enhanced secretion of IL-12 (Cross *et al.* 2001). Taken together the current results indicate that the Th2-inhibitory effects of IFN-γ and IL-12p70 produced by the consortium if replicated *in vivo* via a course of perinatal probiotic supplementation, may be restricted to the early phase of polarisation of immature Th0 cells. Experiments were initiated to explore this by considering the effects of exogenous IL-12p70 on the CBMC response or neutralising IL-12p70 on the PBMC response.

Current results indicate that synergy between IFN-γ and IL-12p70 is vitally important for the imposition of Th1 cytokine responses induced by the consortium. Furthermore, intrinsic immunomodulatory mechanisms operating to limit Th1 type responses during the neonatal stage appear to be operating, and highlight a reasonable explanation for the limited consortium-mediated Th1 response by CBMCs. These observations are in line with the documented literature that Th1 responses are toxic to the placenta and are inhibited by trophoblast-derived IL-10 and progesterone (Krishnan *et al.* 1996; Roth *et al.* 1996; SzekeresBartho *et al.* 1996). At birth, Th1 responses are of a lower magnitude than later in life and newborn $CD4^+$ T cells produce lower IFN-γ levels than adult naïve T cells due to hypermethylation at CpG and non-CpG sites within the IFN-γ promoter (White *et al.* 2002). In the presence of suboptimal CD28 co-stimulation, IL-12 stimulates the production of both

IL-4 and IFN-γ by neonatal $CD4^+$ T lymphocytes, whereas adult cells do not produce IL-4 under similar conditions (Ohshima and Delespesse 1997). Although the current study did not assess the effects of probiotic bacteria on other prominent Th2 cytokines like IL-4 and IL-5, these effects have been studied by others, and despite experimental differences probiotic bacteria have been demonstrated to inhibit IL-4 and IL-5 production by mitogen stimulated PBMC (Pochard *et al.* 2002; Pierre 2002).

The role of the relatively novel pro-inflammatory cytokine IL-17 produced by activated T helper cells (Aarvak *et al.* 1999) has generated much interest in studies of allergic disease and asthma. Therefore the effect of the study consortium on IL-17 production by CBMCs and PBMCs was also considered. Both PHA and SEB significantly induced IL-17 from PBMCs than CBMCs. Interestingly the study consortium potentiated the effects of SEB and PHA for CBMCs but had negligible effect on the response by PBMCs.

3.4.4 Requirement for contact between bacteria and mononuclear cells

Probiotic bacteria may mediate their effects via direct cellular contact or by release of soluble antimicrobial factors that interact with the host immune system. Since the process of probiotic bacterial translocation via the M cell through the gut epithelial barrier is well characterized; transwell experiments were conducted to determine whether cytokine responses induced by the consortium were dependent on direct contact with mononuclear cells and not a secreted immunogenic product of probiotic metabolism. As a result of M cell mediated translocation *in vivo,* probiotic bacteria may encounter macrophages or DCs to stimulate GALT mucosal immunity via induction of suppressive pro-inflammatory immune responses

The current *in vitro* study indicated that direct interaction of the consortium with mononuclear cells was necessary to initiate immunomodulatory signals. A generally accepted paradigm is that dietary consumption of probiotic rich food culminates in interaction of immunoregulatory LAB with cells of the GALT system supported by evidence demonstrating direct interaction of LAB with intra-intestinal lymphoid foci (Cross *et al.* 2001).

Since TNF-α, IL-12p70, IL-1β and IL-10 cytokine production by CBMCs and PBMCs was essentially abolished in the presence of a transwell prohibiting contact between bacteria and mononuclear cell; the current *in vitro* study indicated that direct interaction of the consortium with PBMCs or CBMCs is necessary to initiate immunoregulatory signals. Other studies have demonstrated that direct contact between LAB and intestinal cells is necessary induce expression of cytokine receptors on interferon activated human intestinal cells (Delneste *et al.* 1998).

3.4.5 Evaluation of the principal cellular source of cytokine production from mononuclear cells in response to the consortium

This aspect of the *in vitro* study sought to determine the cellular source of cytokines produced in response to the consortium. A comparison was made of total MNCs preparations that retained their monocyte population, MNC preparations virtually devoid of monocytes and a preparation of cells comprised only of $CD14^+$ monocytes. TNF-α was selected as the cytokine for this part of the study chiefly because it is a marker of heightened immunological activity and a mediator of both Th1 and Th2 cytokine pathways. From these studies, it is clear that monocytes are the main contributors to inflammatory cytokine response to the study consortium. However, whether they are the actual source of the cytokines or are essential to the response made by other non-monocyte cells remains to be determined. There was some cytokine production in the CD14 depleted preparation suggesting that other cell types, in all likelihood dendritic cells, might also contribute to the total cytokine output in response to the study consortium bacteria. Unfortunately, the need for large volumes of umbilical cord blood for this experiment means that the role of neonatal monocytes was outwith the present thesis, but would be an interesting area of research for the future.

The induction of cytokine secretion in response to bacterial cell walls of the intestinal microbiota is serum-dependent, and both CD14 dependent and CD14 independent pathways are involved (Chen *et al.* 1999). It has been demonstrated that lactobacilli induce secretion of IFN-γ by human NK cells (Haller *et al.* 2000). This could explain why the CD14 depleted fraction was able to induce a TNF-α cytokine response to the consortium. Further purification of the CD14 depleted fraction into component NK, T and B cell subpopulation might clarify the role of CD14 independent responses to the consortium. This analysis should also be extended to other cytokines since IL-12p70,

IL-10 secretion is produced by B cells and activated APCs, IL-6 is secreted by T cells, and IL-1β are also secreted by T and B lymphocytes, and NK cells (Fitzgerald *et al.* 2001).

3.4.6 Limitations of the study

The present study employed simplified *in vitro* systems in which key players of the mucosal regulatory response are lacking, including the gastrointestinal epithelial cells that regulate interactions between the consortium and monocytes. In this study mononuclear cells offer only a simple model for studying cytokine production by haematopoietic cells in response to lactobacilli and bifidobacteria. *In vivo,* lactobacilli and bifidobacteria strains are not in direct contact with mononuclear cells but instead interact with the gastrointestinal epithelium, where strains can be taken up by macrophages, DCs or M cells at the Peyers patches potentially leading to activation of lymphocytes and monocytes residing in the Peyers patches (McCracken and Lorenz 2001). Mononuclear samples consist of a rich source of monocytes NK, T and B cells; this study offers an adequate model for study of the immunological properties of lactobacilli and bifidobacteria in the GALT. *In vitro* bacteria-host cell studies facilitate the selection of novel probiotics strains for clinical trials. But the role of the consortium on the gastrointestinal epithelium still remains to be elucidated and co-culture with the consortium with a suitable intestinal cell line such as the CaCo2 and HT-29 are suitable candidates for future direction.

Although this *in vitro* study cannot reflect the strain diversity of the microbial ecosystem, we postulated that employing a consortium of four different probiotic strains known to flourish post-natally in the newborn GIT would account for the strain specific immunomodulatory diversity.

The premise that a vast majority of the intestinal microbiota is currently unculturable employing conventional techniques adds further complexity to the probiotic concept, and further opens the door for research defining optimal probiotic consortiums for immunomodulatory purposes in atopic disorder.

3.4.7 Final summary

Post-natal colonisation of the neonatal GIT provides immunomodulatory activity during a critical period in life, when immunoregulatory aberrancies, due to

inadequate microbial colonisation may induce IgE-mediated atopic clinical disease (Isolauri E 2004).

The consortium in the present study induced *in vitro* mononuclear cells to generate a monocyte dependent, dose dependent release of the pro-inflammatory cytokines TNF-α, IL-6, IL-1β, IL-17, IL-12p70, IFN-γ, the anti-inflammatory cytokines IL-10 and the regulatory cytokine TGF-β1. The observed cytokine responses were dependent on direct contact between the consortium and mononuclear cells.

Interestingly, CBMCs displayed distinctly attenuated cytokine induction kinetics in comparison to PBMCs, and were impaired in their ability to produce IL-12p70 and IL-10 following incubation with the consortium. These two observations are likely intimately linked and reinforce the notion of the under-developed Th1scheme of newborn immunity that renders newborns susceptible to intracellular infections, for which Th1-cell mediated immunity is protective (Siegrist 2001; Adkins *et al.* 2004; Marchant and Goldman 2005).

This is one of few documented studies assessing the kinetics of cytokine production from CBMCs in response to a consortium of probiotic strains at a time when neonatal Th1 immunity is underdeveloped. Newborns exhibit an inherent immunological naivety at mounting Th1 immune responses, and a propensity to respond to antigenic stimuli with a Th2 biased scheme of immunity. Generally, superior Th1 cytokine inducing kinetics in response to the consortium was observed in PBMC samples compared to CBMC samples with all cytokines measured. This can be in part explained by differences in developmental stages and maturational differences between newborn and adult immunity,
In summary, the immunomodulatory efficacy of the consortium is in line with numerous *in vitro* studies highlighting that Gram-positive probiotic bacteria of lactobacilli and bifidobacteria origin actively induced the expression of a range of potent pro-inflammatory and regulatory cytokines, while down-regulating Th2 responses from mononuclear cells *in vitro* (Miettinen 1996; Miettinen 1998; Hessle 1999; Haller 2000; Shida 2006; Helwig *et al.* 2006).

The pro-inflammatory cytokines TNF-α and IFN-γ, mediate the inflammatory response, whereas anti-inflammatory cytokines such as IL-10, are negative regulators

(Chen *et al.* 1999). The study consortium was able to engage both inflammatory and anti-inflammatory arms of the immune response albeit to a greater extent in adults. This discrepancy in newborn versus adult responses to the consortium implicates engagement of immunological pathways leading to a more subtle attenuated inflammation in the newborn to probiotic bacteria compared to the adult. At the gastrointestinal mucosal level this mode of inflammation is postulated to encourage a regulatory scheme of immunity (**Figure 3.12**).

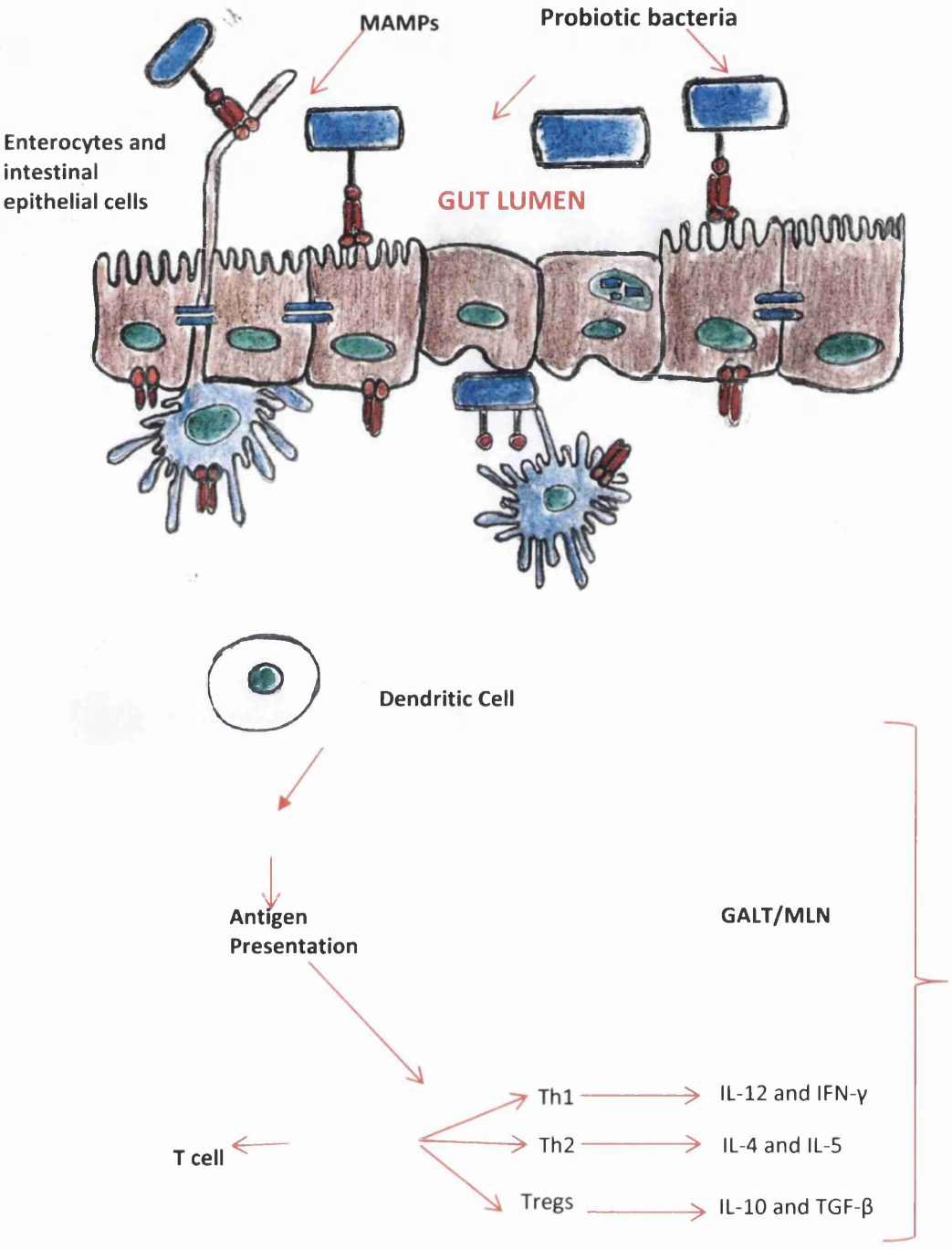


Figure 3.12 Interaction of probiotic bacteria with intestinal epithelial cells and dendritic cells from GALT or MLN. Diagram hand-drawn and adapted from (Lebeer *et al.* 2010). Probiotic bacteria are interactive with intestinal epithelial cells (IECs) and dendritic cells (DCs), by virtue of a dynamic mucus layer where they indiscriminately encounter DCs through two routes: (1) DCs residing in the lamina propria which sample luminal bacterial antigens by passing their dendrite processed between IECs into the gut lumen, and (2) DCs interacting directly with probiotic bacteria that gained access to the GALT via a specialized epithelial cells, termed M cells. The interaction of DCs or enterocytes with MAMPs present on the surface macromolecules of probiotic bacteria induces a specific molecular cytokine response via signal transduction through the TLRs, which interpret probiotic signals via activation of cytokine transcriptional pathways. Other important responses of DCs against probiotics include the production of major histocompatibility complex molecules for antigen presentation, and co-stimulatory molecules that polarize T cells into T helper or $CD4^{+}CD25^{+}$ regulatory T cells in the mesenteric lymph nodes or sub epithelial dome of the GALT (Lebeer *et al.* 2010).

At present, the availability of comparative data on the ability of probiotic strains to induce cytokine production within a similar *in vitro* experimental system, especially models employing CBMCs, are limited (Nier 2005; Foligne *et al.* 2007). However further studies involving co-culturing probiotics with mononuclear cells are coming to the forefront with a view to: (1) Determine mechanisms of probiotic immunomodulatory action to regulate immune responses from allergen-stimulated mononuclear cells extracted from allergic patients, and (2) select probiotics by virtue of their *in vitro* capacity to produce anti-inflammatory cytokines such as IL-10, implicated for its regulatory role in dysregulated immune disorders (Cross *et al.* 2001).

In light of the *in vitro* data, the present study demonstrates that the consortium can boost *in vitro* immune function in a way that can potentially lessen the symptoms of allergic disease in an *in vivo* scenario due to the ability of the consortium to down regulate PHA and SEB induced (Th2) IL-13 while up-regulating (Th1) IFN-γ responses. Furthermore, the results were consistent with previous data by Pierre (2002), who noted that LAB were capable of potentiating mitogen induced Th1 cytokine responses by mononuclear cells, while down regulating mitogen-induced Th2 responses from the same cells.

The question of the net effect of the consequences of immune stimulation provided by the consortium *in vitro,* and whether the consortium can potentially promote suppressive Th2 antagonising immunomodulatory effects in sufferers of IgE mediated allergies, remains unanswered on the basis of the *in vitro* data presented.

However, the *in vitro* immunomodulatory characteristics of the consortium will be used to forecast potential mechanisms of immunomodulatory action in allergic disorder.

Characterisation of interactions between commensal bacteria and the GALT provides the basis for understanding the role of the gut microbiota in achieving a homeostatic disease-free state in the host. This *in vitro* study demonstrated that the consortium exerts an immunosuppressive function via induction of the suppressive cytokines IL-10 and TGF-β1 likely produced by regulatory T cells and T helper cells, which contribute to the anti-inflammatory tone of the gastrointestinal immune system.

Chapter 4: Probiotics in the prevention of atopic disorder: A randomised controlled trial

4.1 Background

Atopic disorder encompasses a broad range of diseases including atopic eczema, allergic rhinitis and asthma. These disorders are marked by a genetic propensity to develop allergen specific immunoglobulin E antibodies following allergen exposure. Upon resolution of an allergic response, atopic sensitisation to a particular allergen may ensue, to this end eczema and asthma early in life has been associated with atopic sensitisation and persistent disease (Arshad *et al.* 2001; Wüthrich *et al.* 2003; Illi *et al.* 2006). Despite the heterogeneity in atopic disorders, as evidenced by differences in how they manifest clinically; atopic disorders share a commonality in that they arise from a complex interplay between environmental and genetic factors. This interplay is determined by microbial exposure during the perinatal period, and genetically determined differences in the immune response mounted by non-atopic and atopic individuals towards allergens (Bieber 2010). Factors that cause the initiation and progression of atopic disorder in early life remain poorly understood. However the hygiene hypothesis proposed by Strachan (1989) based on observations of increased atopy in smaller families postulates that reduced exposure to childhood infections causes aberrant immunological responses to allergens (Okada et al. 2010). Additionally the microfloral hypothesis posits that the intestinal microbiota provides

a crucial source of post-natal immune stimulation regulating host genes of innate and adaptive immunity. Furthermore inadequate early gut colonisation has been associated with the development of atopic disease (Shreiner *et al.* 2008: Yoo *et al.* 2007) thus adding a further layer of complexity to ideas introduced in the hygiene hypothesis. The human microbiota therefore has a vital immunomodulatory influence upon optimal establishment of mucosal and skin barrier integrity against allergen threats (see 1.5). In support of this premise probiotic bacteria of the intestinal tract is postulated to confer a health benefit on the host when administered in adequate amounts as a food supplement. The immunomodulatory mechanisms by which perinatal probiotic supplementation might work *in vivo* to prevent atopic disorder remain largely unclear. However it is hypothesised that a two-fold mode of action might exist involving: (1) modulation of the developing immune system away from development of IgE sensitisation. And (2) reduced exposure to allergens through improved epithelial barrier function, a downstream immunological consequence mediated by the Th1, cytokine, activating potential of specific probiotic bacterial strains. It is the differentiative effects of the Th1 cytokines that are implicated in inhibiting atopic sensitisation, due to their inhibitory effect on the Th2 cytokines involved in the differentiation IgE producing B lymphocytes (Flohr 2011).

4.1 Aim of the study

This part of the thesis evaluated the effect of the probiotic study consortium or an inert placebo, administered to mother-infant dyads during the perinatal period on clinical and laboratory manifestations of atopy at age 0–2 years.

4.2 Rationale and Methodology behind the study

4.2.1 Participants

Allen *et al.* (2014) recruited women aged 16 years or more intending to give birth at Singleton Hospital, with a normal singleton pregnancy. Women with known adverse conditions likely to affect them or the fetus, during the pregnancy were excluded from the study. Allen *et al.* (2014) preferentially intended to recruit newborns at a 'high-risk' of developing atopy, but in practice the authors report that comprehensive details of a familial history atopy was at times difficult to obtain in 80 out of 454 participants on the trial who were believed to be not at risk of developing atopy. Similarly to the works of Kalliomaki *et al.* (2001), newborns with a first degree

relative with either asthma or eczema as diagnosed by a health care professional, or allergic rhinitis treated by a doctor were considered at 'high-risk' of developing atopy, and therefore preferentially selected for the study.

4.2.2 Randomisation

Allen *et al.* (2014) allocated pregnant women at 36 weeks gestation to either the probiotic or placebo arm of the study according to a computer-generated, random sequence generated by the industrial partner (Cultech). The random sequence was held independently of the research team. Study participants were provided with intervention packs corresponding to either the probiotic or placebo arm of the study (Allen *et al.* 2014).

4.2.3 Study consortium

The probiotic intervention comprised a vegetarian capsule containing *Lactobacillus salivarius* CUL61 (National Collection of Industrial, Food and Marine Bacteria (NCIMB) 30211) 6.25×109 colony forming units (CFUs), *Lactobacillus paracasei* CUL08 (NCIMB 30154) 1.25×109 CFUs, *Bifidobacterium animalis* subspecies *lactis* CUL34 (NCIMB 30172) 1.25×109 CFUs and *Bifidobacterium bifidum* CUL20 (NCIMB 30153) 1.25×109 CFUs as a freeze-dried powder. Allen *et al.* (2014) confirmed organism identity at the species and strain levels by 16S rRNA gene sequencing, rep PCR fingerprinting and cluster analysis, and Random Amplified Polymorphic DNA typing. Furthermore no live bacteria were identified in the placebo capsules, conforming with the random allocation sequence.

During the supplemental study Allen *et al.* (2014) posit a rationale for the use of a multispecies probiotic preparation. It was viewed that a multispecies supplement might have a greater protective efficacy against atopic disease rather than either a single species, or single organism preparation (Timmerman et al. 2004). Further Allen *et al.* (2014) go on to describe that the strains in the trial supplement from the genus Lactobacillus have been intentionally selected; and previously evaluated in clinical trials by Kalliomaki *et al.* (2001) studying the role of probiotic bacteria in the prevention of eczema. Additionally organisms were also selected on the basis of their *in vitro* capacity to initiate Th1 cytokine responses by mononuclear cells (personal

communication; Dr S. Plummer 2006). Such responses *in vitro* would be consistent with protection against an allergic immunological microenvironment if replicated *in vivo* due to the inhibitory nature of Th1 cytokine responses on the Th2 cytokine responses that orchestrate the allergic response. The anti-allergy immunomodulatory effects of the study consortium were further corroborated in chapter 3 of this thesis which studied the immunomodulatory effects of the study consortium on cytokine production by umbilical cord blood and adult peripheral blood mononuclear cells *in vitro*. (See 3.4.1 for a comprehensive analysis).

4.2.4 Capsule administration

Women in the placebo arm received capsules of identical appearance containing maltodextrin powder. The dose in both arms was one capsule daily from 36 weeks gestation until delivery. Infants received the same capsules as their mother once daily from birth to age 6 months (Allen *et al.* 2014).

Women either administered the capsule by mouth or sprinkled the contents onto food. The contents of the capsule were sprinkled directly into the infant's open mouth or mixed with expressed breast milk or formula feed. Additionally mothers were informed not to consume or give to their infants any commercially available products similar in nature to the study consortium. Other commercially available probiotics or live yoghurts were therefore prohibited for consumption by recruits on the trial (Allen *et al.* 2014). Viability of the bacteria in the active preparation was consistent with the storage conditions confirming no significant deterioration in the product (Allen *et al.* 2014).

4.2.5 Data collection and allergen testing

Allen *et al.* (2014) collected demographic information and possible risk factors for atopy during the initial recruitment phase. Follow-up questionnaires were scheduled every 6 weeks to age 6 months, and also at 1 and 2 years of age (Allen *et al.* 2014). Questionnaires recorded any atopic disorders diagnosed by health care professionals, treatments received and the occurrence and duration of common skin, respiratory and gastrointestinal symptoms (Allen *et al.* 2014).

Infants were examined by either a clinician or a trained researcher at research clinics, and the severity of eczema was evaluated employing the scoring atopic dermatitis

index (scorad) (Kunz *et al.* 1997). Skin prick tests (SPTs) using common food allergens (cow's milk, hen's egg), aeroallergens (house dust mite, cat dander, grass pollen) and positive (histamine) and negative controls were also performed, with the response to an allergen considered positive if there was a wheal diameter ≥3 mm (Allen *et al.* 2014).

4.2.6 Outcome measures

The primary outcome for the probiotic study was the cumulative frequency of diagnosed eczema at 2 year follow-up (Allen *et al* 2014). Eczema was defined as an itchy rash affecting the face, scalp or extensor surfaces of the limbs in infants and flexures in older children and of duration ≥4 weeks and with ≥1 exacerbation by age 24 months (Leung *et al.* 2003). Based on the information from questionnaires Allen *et al* (2014) determined a set of secondary outcomes for the study, these included:

(1) Responses to SPTs.
(2) Atopic eczema defined as eczema with one or more positive SPTs.
(3) Eczema of any duration (whether or not diagnosed by a health professional).
(4) Severity of eczema.
(5) Extent of treatment with topical steroid preparations.
(6) Respiratory symptoms with asthma and allergic rhinitis and
(7) Reported food allergy.

4.2.7 Data management and analysis

Strict blinding of the clinical research teams and laboratory staff regarding participant allocation was maintained until after databases were locked following completion of data collection (Allen *et al.* 2014). The follow-up period was defined by the authors as the time from birth to the age of the last follow-up questionnaire, and was specifically calculated for each infant. Demographic variables, possible risk factors for atopy, and primary and secondary outcomes were also considered and analysed by treatment allocated. Findings for binary outcomes were expressed in odds ratios (ORs) with 95% confidence intervals (CIs). For a detailed description of statistical tests employed see Allen *et al* (2014).

4.2.8 Sample size

Allen *et al.* (2014) aimed to recruit sufficient mother/infant dyads to observe a 50% reduction in the frequency of asthma by age 5 years from 20% to 10% (Kaur *et al.*

2004) the authors anticipated that 40% of infants at increased risk of atopy in the placebo arm would have developed eczema by age 2 years (Kalliomaki *et al.* 2001; Wadonda-Kabondo *et al.* 2003). And that a total of 154 infants in each arm of the study would be sufficient to detect a 50% reduction in eczema frequency from 40% in the placebo arm to 20% in the probiotic arm with 90% power at the 1% significance level.

4.3 Results

Allen *et al.* (2014) report that recruitment began in May 2005 with the last newborn recruited to the study becoming 2 years old in November 2009, for the last scheduled follow up visit at 2 years old. Of 1419 women attending antenatal clinics who had expressed an interest in the study, 454 were eventually recruited and randomised according to the study. Demographic variables and possible risk factors for atopy were similar in the 220 women randomised to the probiotic arm and 234 to the placebo arm. Maternal smoking during pregnancy, keeping pets and houses affected by damp or mould was common in both arms. 374 women were carrying an infant at high risk of developing atopy, while 80 women were carrying an infant not at risk of developing atopy. A similar proportion each category of woman was recruited to each study arm see table 4.1 by (Allen *et al.* 2014).

Table 4.1 Demographic characteristics and possible risk factors for atopy at baseline

Variable	Probiotic arm*	Placebo arm*
Socioeconomic status (Townsend rank; N; median, IQR)	220; 949 (333–1514)	234; 864 (330–1558)
Mother smoked during pregnancy	41/205 (20.0%)	47/218 (21.6%)
Vaginal delivery	152/216 (70.4%)	157/232 (67.7%)
House		
Cat, dog, rodent or bird kept indoors	112/220 (50.9%)	120/234 (51.3%)
Damp and/or mould as reported by participants	59/217 (27.2%)	56/232 (24.1%)
No. households with		
1 adult	10/220 (4.6%)	9/234 (3.9%)
≥3 adults	20/220 (9.1%)	25/234 (10.7%)
no other children	81/220 (36.8%)	93/234 (39.7%)
≥3 other children	16/220 (7.3%)	18/234 (7.7%)
No. infants at increased risk of atopy†	197/220 (89.6%)	205/234 (87.6%)

This table gives the number of participants (%) unless otherwise stated.
*Denominator varies according to information provided by parents/carers and compliance with follow-up.
†Defined as first degree relative with either asthma or eczema diagnosed by a health professional or allergic rhinitis treated by a doctor.

Allen *et al.* (2014) also reported potential modifying factors for atopy during follow-up periods, such as feeding practice, exposure to other people and antibiotic usage was similar in both arms of the study (table 4.3.2).

Table 4.2 Modulating factors that could potentially influence the atopic status of study participants during follow-up

Variable	Probiotic arm	Placebo arm
Breast fed (full or partial)—any duration	49/191 (25.7%)	47/205 (22.9%)
Age last breast fed in months (N; median, (IQR))	185; 1 [0–7]	190; 1 [0–5]
Attended child-minder in first 12 months	10/163 (6.1%)	16/172 (9.3%)
Attended nursery in first 12 months	65/162 (40.1%)	61/170 (35.9%)
Any oral/systemic antibiotics	155/214 (72.4%)	154/225 (68.4%)

This table gives the number of participants (%) unless otherwise stated.

The authors also report that completion of questionnaires and attendance at research clinics was similar in the two arms (figure 4.1) with the median (IQR) age at follow-up being 2.11 years (2.01–2.28 years) in the probiotic arm and 2.09 years (2.01–2.24 years) in the placebo arm (Allen *et al.* 2014).

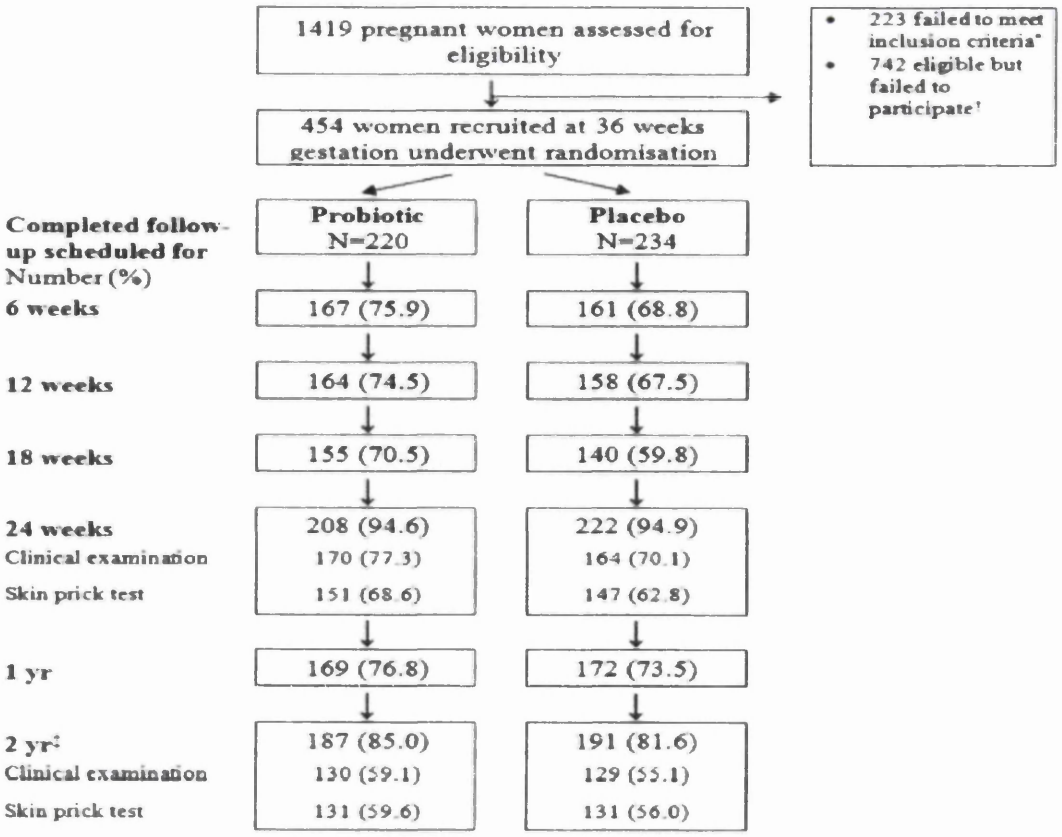


Figure 4.1 Trial profile of Allen *et al.* (2014)

*Reasons for exclusion: pregnancy complication (n=110); presented after 36 weeks gestation or at the end of the recruitment period and before 36 weeks gestation (n=83); multiple pregnancy (n=14); contacted again after recruitment terminated (n=9); previous infant participated in trial (n=6); unwilling to stop current probiotic (n=1). †Reasons for failure to participate: lost contact with research team (n=288); not sufficiently interested or disliked tests (n=184); concern that trial was "too much to take on" (n=129); unwillingness to take the investigation products (n=85); not prepared to be allocated to the placebo arm (n=21); developed a medical condition (n=16); language difficulties (10); not willing for this child to be treated differently from previous child (n=7); bereavement (n=2). ‡Follow-up was often delayed; includes children seen up to, but not including, age 3 years (Allen et al. 2014).

4.3.1 Primary outcome measure

Allen *et al.* (2014) demonstrated that the cumulative frequency of diagnosed eczema at 2 years of age was similar in the probiotic (73/214; 34.1%) and placebo arms

(72/222, 32.4%) of the study. Their result was further supported by observations that the cumulative frequency of parental reported eczema at 2 years of age, was also similar in the two arms. The severity of eczema and duration of rash tantamount with the use of alleviate topical steroids for eczema was also similar in the two arms (Allen *et al.* 2014).

4.3.2 Secondary outcome measures

Allen *et al.* (2014) demonstrated that positive SPTs to one or more common allergens at either age 6 months or 2 years were significantly less frequent in the probiotic arm (OR 0.52; p=0.0360) in comparison to the placebo arm. The authors observed that reduced skin prick responses in the probiotic arm were mainly to food allergens (cow's milk and egg proteins) and statistically significant differences were already apparent at age 6 months (Allen *et al.* 2014). Interestingly the authors discovered that sensitisation to aeroallergens (house dust mite, cat dander and grass pollen) occurred mainly after 6 months and was similar in the two arms. Interestingly the authors found that diagnosed atopic eczema at 2 years was significantly less frequent in the probiotic arm (OR 0.40; p=0.024) these results were consistent with comparisons of early diagnoses of atopic eczema at 6 months of age where infants recruited to the probiotic arm of the study presented less frequently (4/151) with atopic eczema when compared to infants in the placebo group (13/147). This observation reached statistical significance (P=0.021) (Allen *et al.* 2014). For a comprehensive summary of all secondary outcomes please refer to table 4.3.

Table 4.3. Secondary Outcomes of the probiotic study

Variable	Probiotic arm	Placebo arm	OR (95% CI)	p Value*
SPT† positive at 6 m	6/151 (3.97%)	16/147 (10.88%)	0.34 (0.13 to 0.89)	0.023
▸ cow's milk	0/148 (0.0%)	5/147 (3.40%)	-	0.030*
▸ egg	5/148 (3.4%)	14/147 (9.5%)	0.33 (0.11 to 0.95)	0.032
▸ house dust mite	1/151 (0.66%)	0/147 (0.0%)	-	0.51*
▸ cat	0/151 (0.0%)	2/145 (1.4%)	-	0.24*
▸ grass	1/150 (0.67%)	0/147 (0.0%)	-	0.49*
SPT† positive at either 6 m or 2 yrs	18/171 (10.5%)	32/173 (18.5%)	0.52 (0.28 to 0.98)	0.036
▸ cow's milk	1/171 (0.6%)	6/173 (3.5%)	0.16 (0.02 to 1.4)	0.12*
▸ egg	9/171 (5.3%)	19/173 (11.0%)	0.45 (0.2 to 1.0)	0.052
▸ house dust mite	9/171 (5.3%)	11/173 (6.4%)	0.82 (0.3 to 2.0)	0.66
▸ cat	3/171 (1.8%)	7/173 (4.0%)	0.42 (0.1 to 1.7)	0.20
▸ grass	2/171 (1.2%)	2/173 (1.2%)	1.0 (0.14 to 7.2)	0.99*
Skin				
▸ Atopic eczema at 6 m	4/151 (2.7%)	13/147 (8.8%)	0.28 (0.089 to 0.88)	0.021
▸ Severity of eczema at 6 m clinic‡; median, (IQR)	14.3 (7.5–17.9)	14.4 (10.6–24.9)	-	0.46
▸ Atopic eczema at 2 yrs	9/171 (5.3%)	21/173 (12.1%)	0.40 (0.18 to 0.91)	0.024
▸ Severity of eczema at 2 yr clinic‡; median (IQR)	11.1 (7.2–20.1)	14.2 (7.2–14.2)	-	0.85
▸ All reported eczema§,¶	119/214 (55.6%)	132/226 (58.4%)	0.90 (0.61 to 1.3)	0.55
▸ Received topical steroid preparation	30/214 (14.0%)	40/226 (17.7%)	0.76 (0.45 to 1.2)	0.29
Respiratory				
▸ All reported asthma¶	23/171 (11.9%)	20/179 (10.0%)	1.2 (0.63 to 2.3)	0.57
▸ Night-time cough	156/214 (72.9%)	164/226 (72.6%)	1.0 (0.67 to 1.5)	0.93
▸ Night-time or daytime cough	178/214 (83.2%)	188/226 (83.2%)	1.0 (0.6 to 1.6)	0.99
▸ Wheezing without symptoms of a virus infection	50/214 (23.4%)	55/171 (24.3%)	0.95 (0.61 to 1.47)	0.81
▸ Inhaled bronchodilator or steroid	26/214 (12.1%)	27/226 (11.9%)	1.0 (0.57 to 1.8)	0.94
▸ Allergic rhinitis¶	10/190 (5.3%)	10/201 (5.0%)	1.1 (0.43 to 2.6)	0.90
▸ Sneezing and/or snuffling	207/214 (96.7%)	212/226 (93.8%)	1.95 (0.77 to 4.93)	0.15
Any reported food allergy	22/200 (11.0%)	31/204 (15.2%)	0.69 (0.38 to 1.2)	0.21

This table gives the number of participants (%) unless otherwise stated.

χ^2 test for contingency tables or Fisher's exact test where expected values in cells were <5.

†SPT valid if diameter of wheal for the positive control was ≥1 mm than diameter for the negative control; SPT positive if diameter of wheal for antigen was ≥3 mm than diameter for negative control.

‡SCORAD index[19].

§Of any duration.

¶Whether or not diagnosed by a health professional.

SCORAD, scoring atopic dermatitis; SPT, skin prick test.

4.3.3 Compliance

Factors determining compliance within the probiotic trial were published and discussed thoroughly by Jordan *et al.* (2013). Unused capsules returned by participants from the probiotic and placebo arms for compliance monitoring were tested by an independent laboratory. Allen *et al.* (2014) discovered that the Median (IQR) compliance with the trial interventions in pregnant women was good, and was 20 (12–28) days in the probiotic arm and 20 (12–28) days in the placebo arm. However in infants compliance was not so good, the corresponding values were 106 (30–141) days in the probiotic arm and 103 (11–151) days in the placebo arm.

4.4 Discussion

Despite promise shown *in vitro* by strains of the study consortium to elicit Th1 cytokines (TNF-α, IFN-γ and IL-12p70) of a pro-inflammatory tone and thereby inhibit the production of the allergy accelerating cytokines involved in the manifestation of atopic disorders such as atopic eczema (Chapter 3). The findings of Allen *et al.* (2014) do not definitively support perinatal administration of a high-dose consortium of probiotic bacteria for the prevention of eczema in early childhood. This result was unexpected, and contrasted with initial hypothesises introduced in chapter 3, suggesting that the *in vitro* potential for the study consortium to initiate pro-inflammatory cytokine responses, antagonistic to the development of allergy driving Th2 cytokines could occur *in vivo* as a downstream immunological effect perinatal probiotic supplementation. Interestingly Allen *et al.* (2014) discovered that perinatal probiotic administration was associated with a reduced frequency in IgE-mediated sensitivity to food antigens and atopic eczema. However the immunological mechanisms responsible for the observed reduction in IgE-mediated atopic sensitisation require further elucidation.

4.4.1 Limitations of the study

The findings of Allen *et al.* (2014) that the study probiotic did not affect the frequency of eczema is inconsistent with a meta-analysis of 13 randomised, placebo-controlled trials (3092 infants/children), where probiotic administration was associated with modest reductions in the frequency of eczema (Pelucchi *et al.* 2012). However Allen *et al.* (2014) do acknowledge several weakness of their study. For instance the authors had intended to evaluate clinical outcomes in infants at increased

risk of atopy, but in practice the atopy status was not always straightforward to accurately determine at recruitment. This meant that approximately 18% of the trial-cohort were non-atopic. However the frequency of atopy and effects of the probiotic were similar in infants with and without a first degree relative with atopy (Allen *et al* 2014).

Allen *et al* (2014) also mention that they had expected that administering a novel intervention to healthy young infants would be laborious and although they cite that compliance with the trial interventions in mothers was good, compliance was far lower in infants. Mothers returning to work were also cited as a reason for missed or delayed follow-up questionnaires and study clinic attendances.

Another point of consideration is that there is a paucity of studies assessing how potential probiotic bacterial strains survive in the newborn GIT when employed as multi-species supplements. For instance the efficacy of GIT colonisation of each strain in a multi-species supplement is not often reported in a number of probiotic intervention studies. Furthermore it is not known whether strains of the multi-species supplement have to compete with each other in order to survive in the GIT, thereby reducing the overall effectiveness of the supplement.

Allen *et al.* (2014) observed that a reduced frequency of skin prick sensitivity to food allergens and eczema in children with demonstrated allergic sensitivity in the probiotic arm are consistent with those of a meta-analysis (20 cohorts; 4031 participants, including our findings). In these meta-analyses probiotic administration during infancy and childhood was associated with reduced serum IgE and atopic sensitisation to food or inhalant allergens (Ealzab 2013). The main finding in the study of Allen *et al.* (2014) was that administration of probiotics in early life has a role in the prevention of atopic sensitisation. With the findings of the probiotic trial at the forefront the tone is now set to interpret the result of the study of Allen *et al.* (2014) in the context of the immunological outcomes of the study. These outcomes are concerned with understanding how the study consortium might impact upon the underlying immunological mechanisms involved in the establishment of atopic sensitization, this is explored in chapters 5 and 6.

Chapter 5: *Ex vivo* effects of perinatal probiotic supplementation on cytokine responses by polyclonally stimulated umbilical cord blood mononuclear cells, and 6 month, infant peripheral blood mononuclear cells.

5.1 Background

Cytokine responses by lymphocytes form the cornerstone immune-transmission network of adaptive immunity and play essential roles in mediating tolerogenic immune responses, mediated against both the intestinal microbiota and ubiquitously occurring allergens (Paul and Seder 1994). By analysing *ex vivo* cytokine induction patterns of both mitogen stimulated newborn and infantile mononuclear cells isolated from recruits on the PROBAT trial (described in Chapter 2), this study assessed by ELISA the effects of perinatal probiotic supplementation on age-related maturational changes in cytokine production from birth and 6 months of age. In addition to assessing the impact of the consortium on infantile atopy and subsequent atopic disease, umbilical cord blood was collected at birth and peripheral blood from the infant to assess the immunomodulatory effects of the study consortium on immune function at birth and in early infancy. Results were assessed in relation to our current understanding of the development of immune function during infancy and immune parameters that related to the initiation and maintenance of atopic disease post-natally.

Cytokine responses by CBMCs in response to polyclonaly mitogenic stimuli are likely to be mediated by a population consisting of mainly immature and antigen inexperienced thymocytes. For instance the neonatal T cell repertoire is almost exclusively of the naive phenotype, accounting for 90% of neonatal $CD4^+$ cells and displaying a reduced capacity for cytokine production compared to memory T cells which occur in greater amounts in PBMCs of 6 month old infants (Harris *et al.* 1992).

Data on the baseline cytokine production profiles upon mitogenic stimulation by mononuclear cells obtained in the neonatal period up until 6 months of age are limited (Hartel *et al.* 2005) for both atopic and non-atopic neonates and children. The principal observation arising in the late 70s was that infants and young children demonstrate an attenuated capacity for T cell responses when compared with older

children (Hayward and Lawton 1977; Clerici *et al.* 1993) with neonatal T cell cytokine responses to polyclonal mitogens and superantigens, significantly reduced in early infancy, a discrepancy that extends even to neonatal T cell proliferative responses (Wilson, Westall *et al.* 1986; Lewis *et al.* 1991; Pirenne-Ansart *et al.* 1995; Smart and Kemp 2002). Such differences reinforce the notion of an age-related immune maturation over time and are reflected in changes in cytokine production from the anti-inflammatory, pro-inflammatory and regulatory cytokine families occurring with age.

Imbalances in effector cell responses are postulated to impact upon the pathophysiology of atopy and subsequent atopic disorder (Hartel *et al.* 2005). More specifically the balance of Th1 (IFN-γ), Th2 (IL-4 and IL-13) and regulatory T cell cytokines (IL-10) may determine the development of atopic disease in childhood and probiotics of the microbiota have been demonstrated to influence the levels of these cytokines in both *in vitro* and *in vivo* scenarios, thereby impacting upon the integrity of mucosal immunity (Gillingham and Lescheid 2009).

The manifestation of atopic disease changes throughout childhood, evolving from early infantile eczema to the development of asthma in a progression entitled 'The Atopic March'(Smart and Kemp 2001). It is therefore of considerable interest to

(1) Appreciate differences between the ontogeny of age-related immunological changes in *ex vivo* cytokine production in response to antigen independent stimulation between healthy non-atopic children versus atopic children.

(2) Whether immunological changes giving rise to a scheme of immunity that supports atopic disorder in neonates showing immunological symptoms of atopy can be manipulated at the neonatal stage via a course of probiotic supplementation toward a non-atopic outcome.

Generally a diminished production of the T cell cytokines IL-13, IL-4, IL-10, IL-12 and IFN-γ in the neonate has been described (Wilson *et al.* 1986; Frenkel and Bryson 1987; Vigano *et al.* 1999; Lewis *et al.* 1991; Pirenne-Ansart *et al.* 1995; Chipeta *et al.* 1998; Gasparoni *et al.* 2003; Prescott *et al.* 2003; Marchant and Goldman 2005). Despite the attenuated immune T cell capacity in the newborn, production of IFN-γ

has been demonstrated to increase rapidly throughout early infancy (Chipeta *et al.* 1998), reaching adult levels by 1 year of age in one study Frenkel and Bryson (1987) or by 3 years of age in another study Miyawaki *et al.* (1985). To further support the notion of a limited Th1 immune repertoire in the newborn compared to mature children, a recent study in healthy newborns, infants and young children aged between 1 and 96 months assessing mRNA cytokine and protein expression in response to phorbol 12-myristate 13-actate/ionomycin stimulation demonstrated that intracellular IFN-γ expression increased significantly and progressively with age (Hartel *et al.* 2005). These observations were supported by works of Chipeta *et al.* (1998) and Gasparoni *et al.* (2003) who also noted that IFN-γ-producing T cell populations increased progressively with age, and that a significant negative correlation exists between gestational age and IFN-γ-$CD4^+$ and IL-10- $CD4^+$-producing cells with neonates having a substantially lower percentages of $CD4^+$ and $CD8^+$ IFN-γ producing cells compared to older children. In a different study with infants aged 2–12 months, the number and percentage of T lymphocytes producing IFN-γ and IL-4 was also shown to be increased over the first year of life, but remaining lower than in adults (Buck *et al.* 2002). Similarly (Tang and Kemp 1995) noted that IL-4 release was reduced in neonates versus adults and remained reduced in children under 10 years of age, but increased progressively with age.

Although a positive correlation is thought to exist between gestational age and IL-10-$CD8^+$-producing cells (Gasparoni *et al.* 2003), IL-10 protein expression tended to be regulated on an individual basis during infancy and early childhood (Hartel, Adam *et al.* 2005), while (PHA)-induced IL-13 release in neonates was similar to that of adults (Williams *et al.* 2000). Additionally (Gasparoni *et al.* 2003) measured T lymphocyte cytokine responses to Concanavalin A (ConA) by measuring intracellular IL-4, IFN-γ and IL-10 in 12 very preterm, 12 preterm, 20 term neonates, 10 children and 10 adults and discovered that immunoproliferation to ConA was significantly lower in cord blood than the blood from children or adults. Their observations were confirmed upon analysis of the percentages of lymphocytes occurring in the CD4 and CD8 compartments of which $CD4^+$ lymphocytes was significantly higher in newborns while $CD8^+$ cells were higher at older ages, with a resulting gradual decline of the $CD4^+/CD8^+$ ratio. While the percentage of IL-4-producing cells was higher for $CD8^+$ and lower for $CD4^+$ cells in cord blood than in children and adults reflecting a gradual development of immunity during gestation

and highlighting a significant immaturity of the cellular immune response at birth (Gasparoni *et al.* 2003).

5.1.2 Newborn and infantile responses to experimental stimuli

In humans, IFN-γ is a frequently measured cytokine for characterization of Th1 cytokine responses, while IL-4 and 10 are often employed to characterize Type 2 cytokine responses. The work presented herein utilised umbilical cord blood and 6 month infant peripheral blood from recruits on the PROBAT trial.

Studies of T cell cytokine release have generally employed the lymphocyte mitogen PHA (Miyawaki *et al.* 1985; Frenkel and Bryson 1987; Pirenne-Ansart *et al.* 1995; Tang and Kemp 1995; Vigano *et al.* 1999) and the T cell mitogen/superantigens *staphylococal* enterotoxin B (SEB) (Wilson *et al.* 1986; Campbell and Kemp 1997), as an antigen-independent polyclonal stimulus to activate T cells by virtue of their non-specific binding to the variable chain of the T-cell receptor bearing the major histocompatibility complex class II chains outside the peptide-binding groove. This interaction has been utilised by numerous authors (Tang and Kemp 1995; Campbell, Fryga *et al.* 1999; Smart and Kemp 2001) to induce vigorous T-cell activation and cytokine release (Skov and Baadsgaard 2000).

To examine the age related ontogeny of cytokine release in the presence of prior probiotic supplementation, a global stimulus capable of activation of multiple T cells mimicking an antigenic interaction is beneficial as activation is not dependent on specific sensitivity to individual antigens (Smart and Kemp 2001). Furthermore, the use of SEB as a stimulus is postulated to be of particular relevance when studying cytokine release in atopic subjects since SEB is postulated to play an important role during development of the inflammatory response in atopic disorder (Hoeger *et al.* 1992; Leung *et al.* 1993; Campbell and Kemp 1997; Hofer *et al.* 1999; Skov *et al.* 2000).

The principle approach adopted in this *ex vivo* study was to determine the impact of probiotic supplementation on their contribution to cytokine release by both cord and 6 month infant blood mononuclear cells stimulated with PHA or SEB to provide an insight into the underlying Th1/Th2 cytokine predisposition in response to antigen independent stimulus. Information on how this predisposition alters within a cohort

of newborns who were susceptible to allergy and undergoing probiotic supplementation was also obtained.

This mitogen-lymphocyte interaction has been utilised to examine cytokine imbalances in childhood atopic disease (Rosenfeldt *et al.* 2003; West *et al.* 2009), and was employed in the PROBAT study to examine the effects of probiotic supplementation on *ex vivo* cytokine responses from mononuclear cells isolated from umbilical cord blood and venous blood of 6 month infants recruited to the PROBAT trial. IL-4, IL-10, IL-13 and IFN-γ outputs at birth or at 6 month of age in supplemented individuals were analysed as exemplars of Th1, Th2 and immunoregulatory cytokine responses. The findings of the present study are likely to reflect cytokine release from both $CD4^+$ and $CD8^+$ T cells by SEB, since superantigens stimulate both $CD4^+$ and $CD8^+$ thymocytes from infants (Thulesen *et al.* 1999).

5.1.3 Aim of study

The principal aim of this study was to examine the effects of perinatal probiotic supplementation with the PROBAT consortium on maturation of age-related changes in newborn immune responses occurring from birth to early infancy. Whether or not perinatal probiotic intervention during a 'critical window' of immune development could attenuate the risk of allergen sensitization and associated disease in clinically predisposed allergic neonates was also assessed in terms of the effects of supplementation on *ex vivo* production of the prototypic Th1 (IFN-γ), Th2 (IL-4 and IL-13) and the regulatory cytokine (IL-10) by mononuclear samples isolated from the cord blood of newborn and 6 month old recruits on the PROBAT trial.

To delineate the immunological mechanisms of action by which probiotic supplementation could prove protective against atopic disorder the propensity of PROBAT trial mononuclear cells to mount *ex vivo* cytokine responses to mitogenic stimulation was characterized following induction of polyclonal proliferation of cord blood mononuclear cells and 6 month infant peripheral blood mononuclear cells in response to (SEB- T cell mitogen) or (PHA -Lymphocyte mitogen) or anti CD3/CD28 beads.

Ensuing immune responses as a result of prior mitogenic stimulation where measured in terms of cytokines synthesised from mitogenically stimulated cord

blood mononuclear cell and 6 month blood mononuclear cells cultures. An assessment on whether probiotic supplementation can induce a T helper cytokine profile protective against allergic responses was then made in terms of how probiotic supplementation could affect the balance of the Th1/Th2/Th17 immune axis.

5.2 Rationale and Methodology behind the study

The availability of detailed immunological phenotypes on newborns recruited on the PROBAT trial at birth and at 6 months of age, offered the opportunity to concurrently compare the effects of the consortium on immunological phenotypes measurable *ex vivo* using cord blood **(CB)** and 6 month peripheral blood **(6Mth PB)** versus those measurable in our *in vitro* model. Blood collected during the PROBAT trial provided the opportunity to: (i) compare responses at birth with those at 6 months of age regardless of whether the child was on the placebo or probiotic arm of the study, and (ii) determine the impact of maternal/infant probiotic consumption on the cytokine response at birth and at 6 months of age.

5.2.1 Preparation and culture of mononuclear cells

Mononuclear cells were isolated from umbilical cord blood at birth and peripheral blood from 6 month old infants by density gradient centrifugation (See 2.1.3). Cells (0.5×10^6/500µl) were cultured for 48 hours at 37°C in 5% CO_2-in-air in the presence/absence of PHA (1 µg/ml), anti-CD3/CD28 T cell expansion beads (5 µl/500µl) or SEB (200 ng/ml). Cell free culture supernatants were harvested and stored at -80°C until analysis. Additional experiments utilized MNCs prepared from random umbilical cord blood samples collected after delivery by elective caesarean section as part of other studies in the group and from healthy adult volunteers. MNCs were prepared and treated in the same way.

5.2.2 Cytokine assays

Supernatants were thawed and analysed for IL-4, IL-10, IL-13 or IFN-γ using specific ELISAs according to the manufacturer's instructions as described (See 2.1.4). Cytokine data were analysed as (a) continuous data and (b) dichotomous data (detected or non-detected).

5.2.3 Statistical analysis for chapters 5 and 6

All data were log transformed before analysis. Statistical analyses were performed using SPSS software or Excel (Microsoft). A p-value < 0.05 was regarded as statistically significant. The student t test was employed as there were no pre-defined ideas about what might change. The ex vivo data of chapters 5 and 6 was an exercise in generating hypothesis, rather than testing them therefore p-values were not adjusted for multiple testing. We did not make formal adjustments for multiple testing for two reasons. First, many of the tests would be correlated; therefore an adjustment based on criteria such as Bonferonni would be highly conservative (and possibly lead to a high false negative rate). Second, we has little prior data on expected differences, hence we consider this an exercise in generating new hypotheses about how probiotics might affect a wide spectrum of immune parameters, rather than an exercise in testing pre-determined hypotheses (personal communication with Professor Gravenor 2010). As such, any significant trends that we detect will be confirmed by follow up studies. The difference in the mean level between the probiotic and control groups was investigated with two-sample t-tests. A significance level of 5% was used throughout. Data were presented as box-whisker plots, with mean, median, interquartile range, and (trimmed) maximum and minimum values.

5.3 Results

5.3.1 Age-related changes in cytokine responses from birth to 6 months of age

To determine age-related changes in cytokine responses at birth and at 6 months of age in the presence of the study consortium or placebo, an initial comparison of cytokines responses made to each stimuli employed was made for mononuclear cells prepared from umbilical cord blood (CBMCs); and mononuclear cells prepared from peripheral blood from 6 month old infants (6 month PBMCs) (Figure 4.1).Where possible, age-related changes in cytokine responses to each stimuli were compared in the same subject at birth, and at 6 months of age. Generally production of the Th2 cytokine IL-4 was significantly greater in 6 month PBMCs samples stimulated with anti-CD3/CD28 beads or SEB (both $p < 0.00001$) but not PHA. For IL-10 the bead-induced response was greater in the infants ($p < 0.00001$) but the PHA-induced response was greater in the cord MNCs ($p = 0.006$), with no difference upon

stimulation with SEB. For IL-13, the only difference was for PHA, with the response by CBMCs greater than that by (6 month PBMCs) ($p < 0.00001$). There were no significant differences for IFN-γ.

As the cytokine response at birth and in early infancy is generally reduced compared to that of adults, an alternative way of analysing the data is to consider what proportion of subjects made a detectable response for each cytokine and stimuli combination. This data is summarised in Table 4.1 detailing the proportion of samples up-regulating a cytokine response for each stimuli.

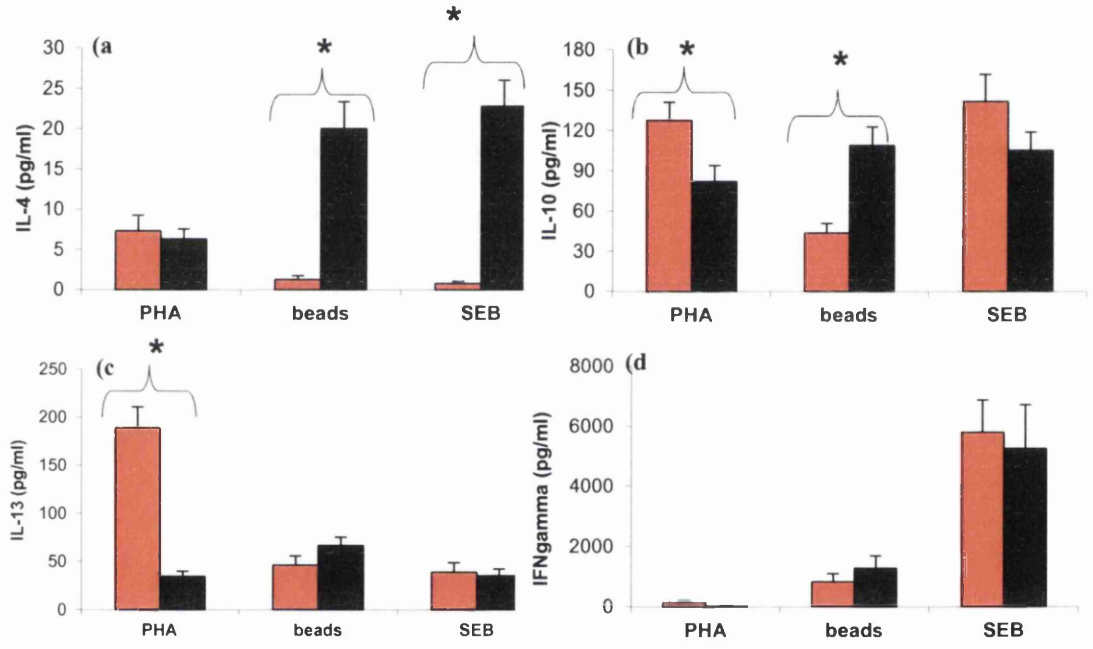


Figure 5.1. Cytokine responses by mononuclear cells from CBMCs (orange) or 6 month infant PBMCs (dark green). Mononuclear cells were prepared by density gradient centrifugation and cultured at 0.5 x 10^6/500µl in the presence of PHA, anti-CD3/CD28 beads (beads) or SEB. Cell free supernatants were harvested and analysed for (a) IL-4, (b) IL-10, (c) IL-13, or (d) IFN-γ using specific ELISAs. Values obtained were corrected for levels in un-stimulated controls (which typically were not detectable) and mean ± SEM determined. * denotes significant difference ($p < 0.05$) between cord and infant.

The IL-4 response to the stimuli was significantly different between the two age groups for all stimuli studied ($p < 0.0001$). There was a statistically significant increase in the proportion of samples that returned a detectable cytokine output at 6 months of age compared to birth (Table 4.1). Other statistically significant differences were: fewer responsive infants for PHA-induced IL-10 ($p < 0.00001$), fewer responsive infants for IL-13 for all stimuli (PHA, $p = 0.0017$; beads, $p = 0.0455$; SEB, $p = 0.0032$), and fewer responsive infants for SEB-stimulated IFN-γ ($p < 0.00001$) (Table 4.1).

Since this analysis was conducted on the whole cohort inclusive, of some matched cord/infant samples, the IL-4 response was considered in more detail to ascertain whether the same pattern of responders/non-responders was observed in samples from the same subject at the two different age points. For the response to beads (Figure 4.2b), there was a clear indication that the levels of IL-4 increased between birth and 6 months of age ($n = 13$); this is not as clear for PHA (Figure 4.2a). There were no matched pairs for SEB stimulation. Levels of IL-4, IL-10, IL-13 and IFN-γ in response to PHA, beads or SEB were determined and when the cytokine was detectable this was recorded as a 'responder' whereas if the cytokine was not-detectable this was recorded as a non-responder. statistically significant results were generated using **Chi-square (χ^2).** $P < 0.05$ was considered statistically significant

Table 5.1. Results of statistical analysis of proportion of responders to each cytokine/stimuli combination.

	IL-4			**IL-10**		
	Cord	**infant**	**p-value**	**cord**	**Infant**	**p-value**
PHA	42/97 (43%)	65/98 (66%)	<0.0001	84/98 (86%)	66/97 (68%)	<0.0001
Beads	16/84 (19%)	72/85 (84%)	<0.0001	58/84 (69%)	64/87 (74%)	0.2543
SEB	11/31 (36%)	31/34 (91%)	<0.0001	29/31 (94%)	32/35 (91%)	0.4846

	IL-13			IFN-γ		
	Cord	**infant**	**p-value**	**cord**	**Infant**	**p-value**
PHA	87/96 (91%)	76/97 (78%)	<0.002	50/96 (52%)	48/97 (50%)	0.548
Beads	78/83 (88%)	67/84 (80%)	<0.046	59/83 (71%)	66/83 (80%)	0.05
SEB	30/30 (100%)	33/36 (92%)	<0.003	30/31 (97%)	22/27 (82%)	<0.0001

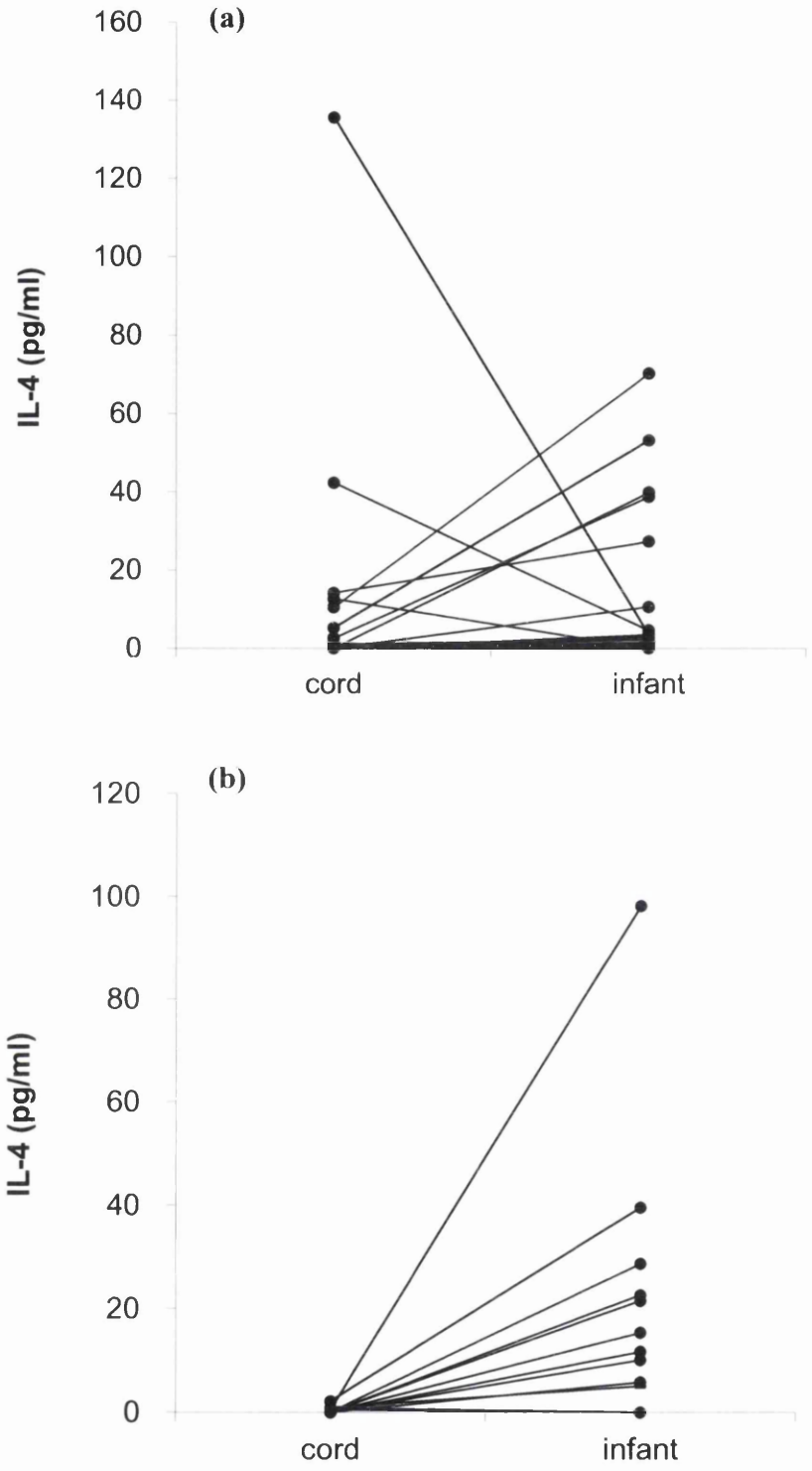


Figure 5.2. IL-4 response by matched pairs of cord blood and 6 month infant peripheral blood MNCs.

MNCs were prepared as previously described and stimulated with (a) PHA or (b) anti-CD3/CD28 beads for 48hr prior to collection of cell free supernatants for later batch analysis of IL-4. Data from matched pairs are shown. PHA, (n = 25); beads, (n = 13).

5.3.2 Cytokine responses by probiotic versus placebo

The number of samples at each study point (**birth** – umbilical cord blood; **infant** – peripheral blood at 6 months of age) for which MNC cultures were available is summarised in Table 4.3. Figures 4.3 to 4.6 show the data for cytokines responses at birth and at 6 months of age for each cytokine and stimuli combination. There were no statistically significant differences.

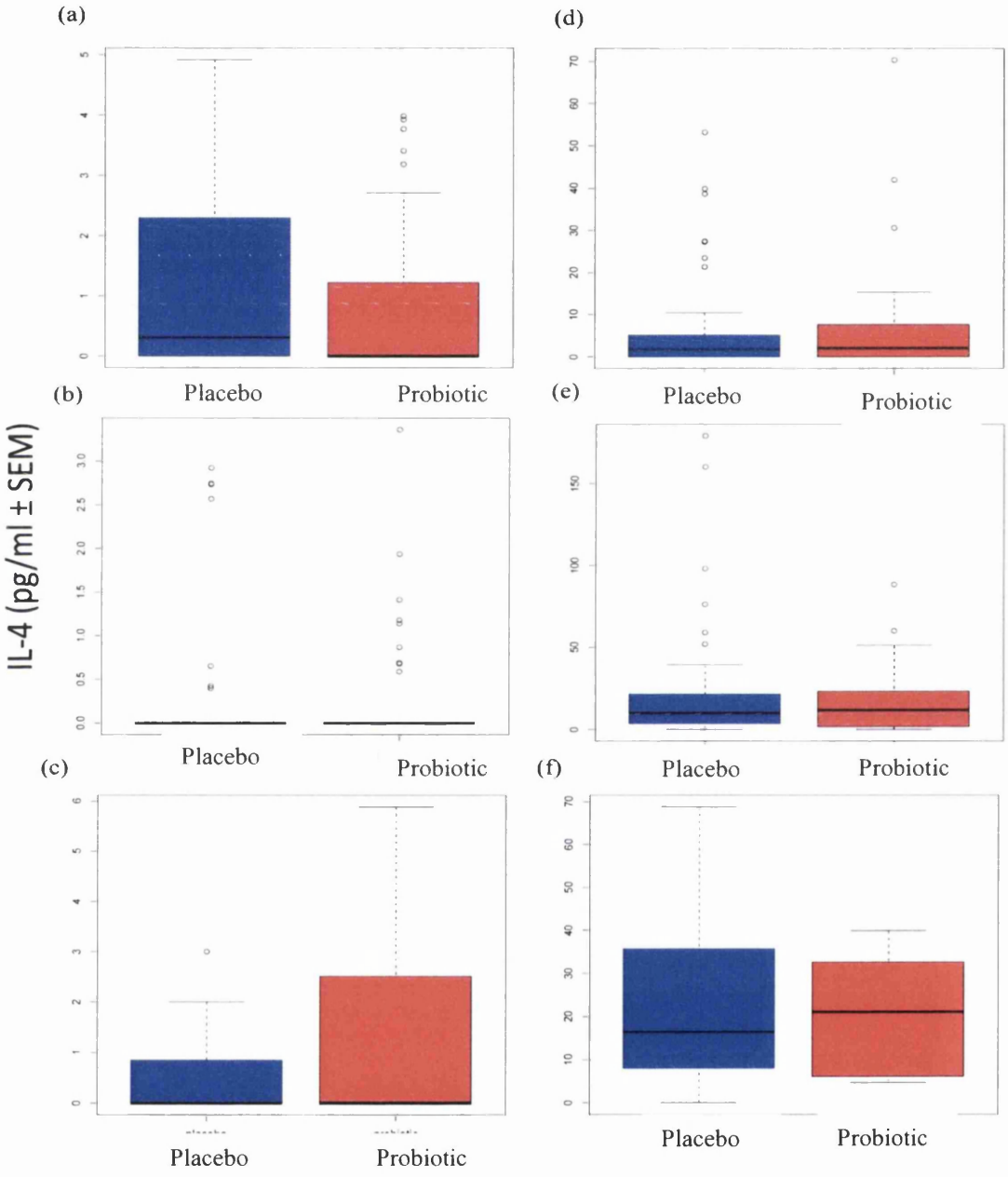


Figure 5.3. IL-4 response by mononuclear cells from participants in the PROBAT trial.

Mononuclear cells were prepared from (a, b, & c) umbilical cord blood and (d, e, & f) peripheral blood of 6 month infants and stimulated with (a & d) PHA, (b & e) anti-CD3/CD28 beads, and (c & f) SEB. IL-4 levels in cell free culture supernatants harvested after 48 hours culture were measured using a specific ELISA. The box signifies the upper and lower quartiles (75% and 25% respectively), and

the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ○ Statistically significant results ($P < 0.05$) were denoted by *

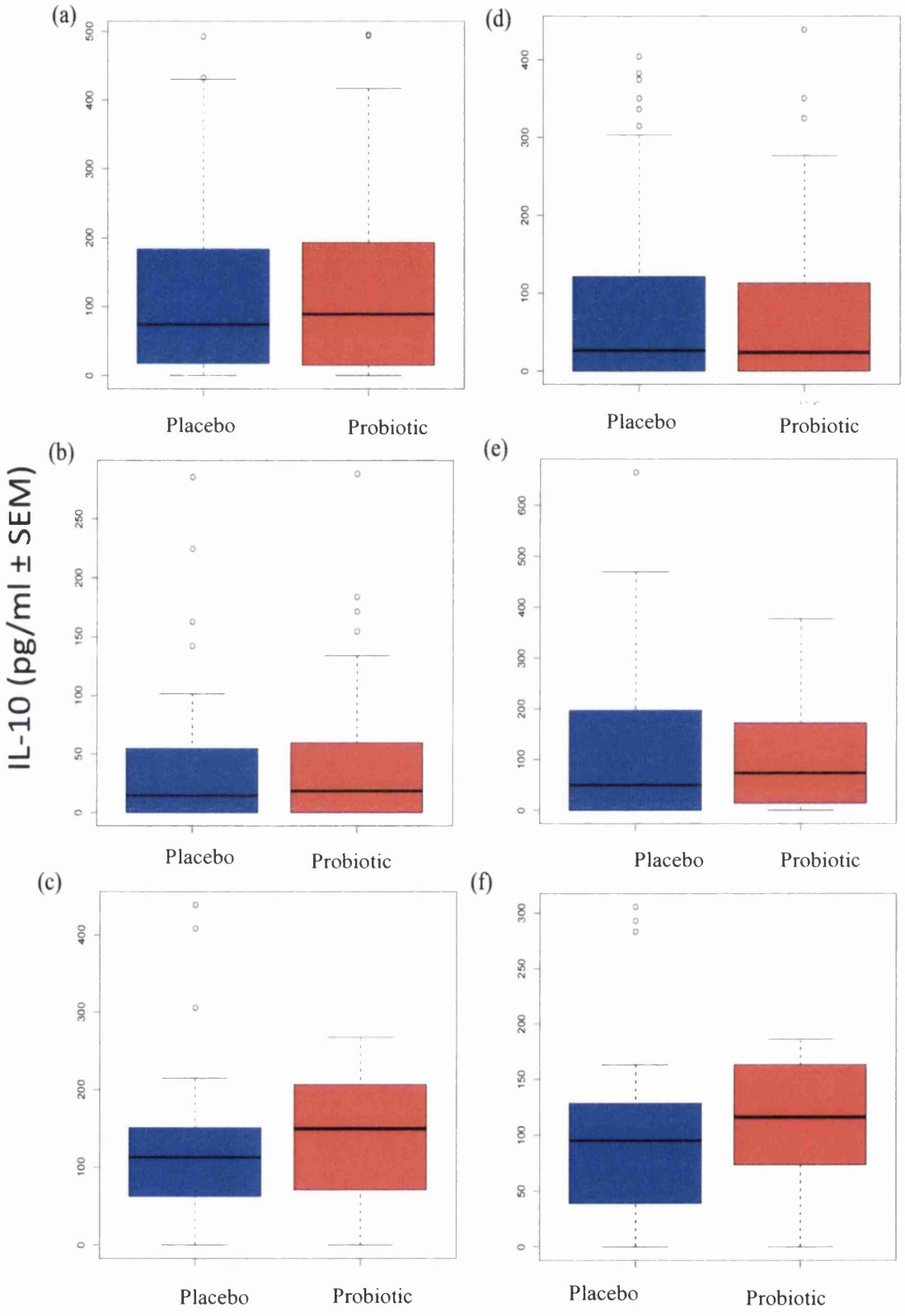


Figure 5.4. IL-10 response by mononuclear cells from participants in the PROBAT trial. Mononuclear cells were prepared from (a, b, & c) umbilical cord blood and (d, e, & f) peripheral blood of 6 month infants and stimulated with (a & d) PHA, (b & e) anti-CD3/CD28 beads, and (c & f) SEB. IL-10 levels in cell free culture supernatants harvested after 48 hours culture were measured

using a specific ELISA. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ○ Statistically significant results ($P < 0.05$) were denoted by *

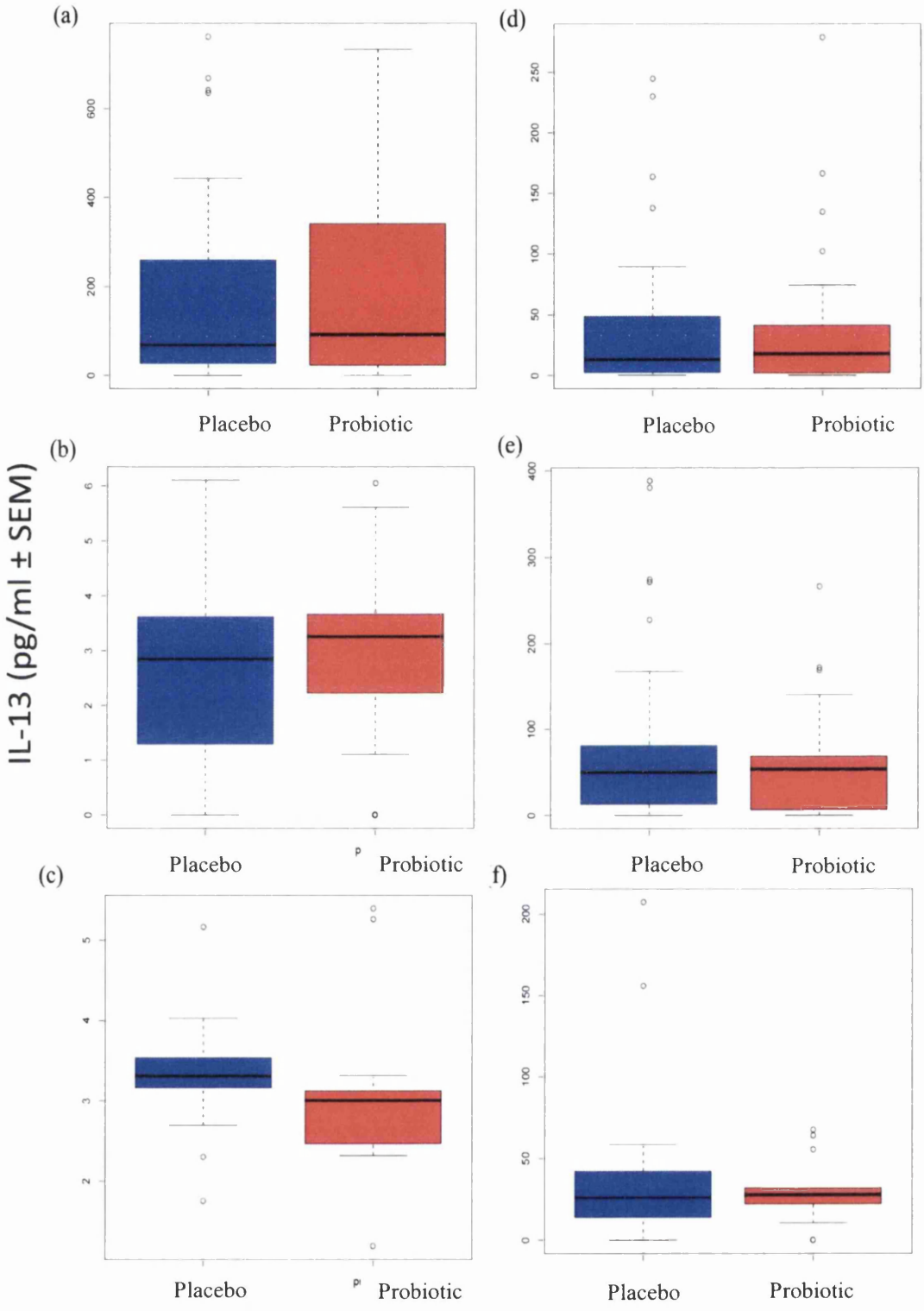


Figure 5.5. IL-13 response by mononuclear cells from participants in the PROBAT trial. Mononuclear cells were prepared from (a, b, & c) umbilical cord blood and (d, e, & f) peripheral blood of 6 month infants and stimulated with (a & d) PHA, (b & e) anti-CD3/CD28 beads, and (c & f)

SEB. IL-13 levels in cell free culture supernatants harvested after 48 hours culture were measured using a specific ELISA. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ○ Statistically significant results ($P < 0.05$) were denoted by *

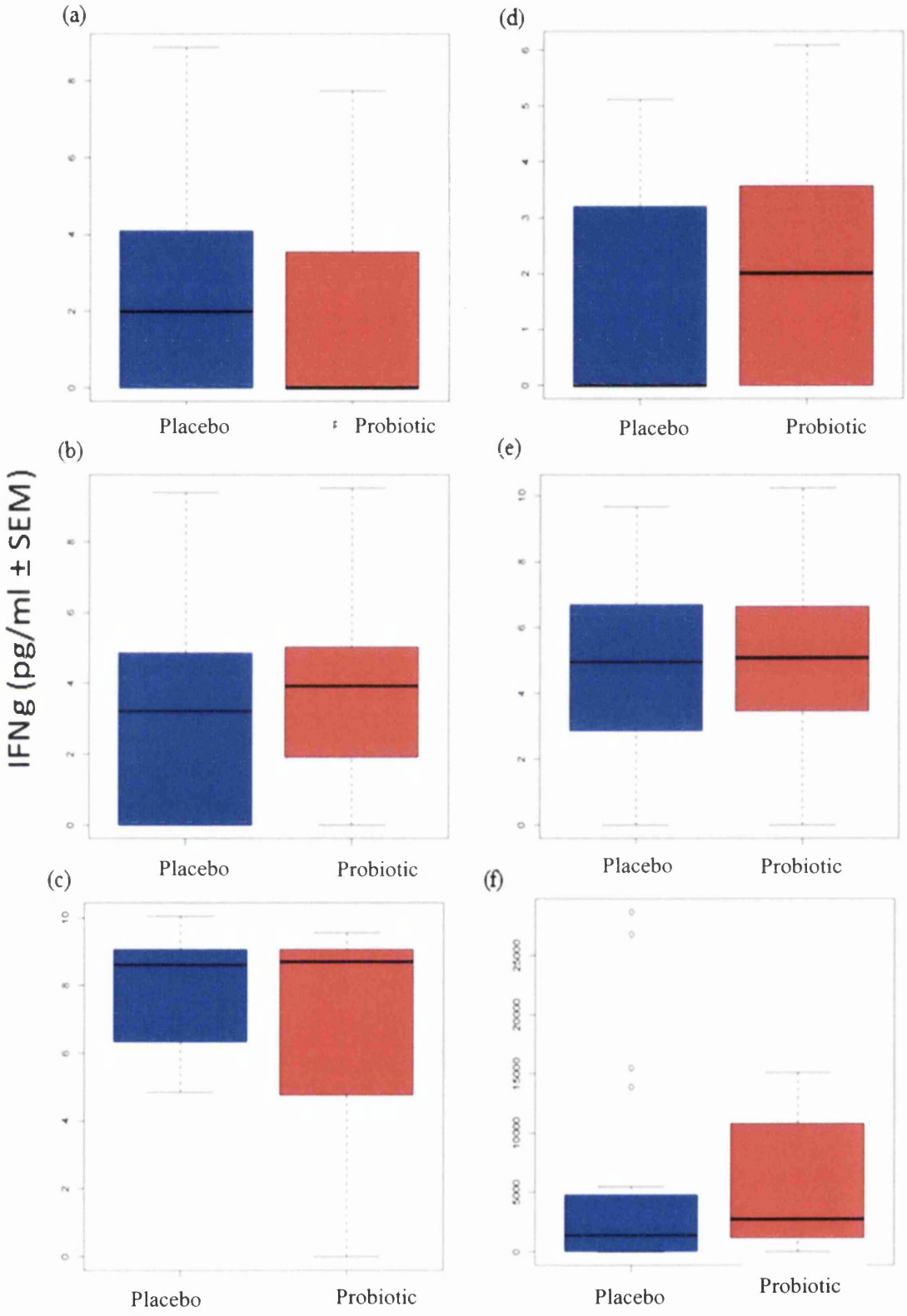


Figure 5.6. IFN-γ response by mononuclear cells from participants in the PROBAT trial.

Mononuclear cells were prepared from (a, b, & c) umbilical cord blood and (d, e, & f) peripheral blood of 6 month infants and stimulated with (a & d) PHA, (b & e) anti-CD3/CD28 beads, and (c & f) SEB. IFN-γ levels in cell free culture supernatants harvested after 48 hours culture were measured using a specific ELISA. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted

5.3.3 IL-17 and IL-22 production in early infancy

Since completing the cytokine analyses shown above, there has been an increased interest in Th17 cells that have as their secreted prototypic cytokine, IL-17. These cells also secrete IL-22. Before determining whether analysis of MNCs from the PROBAT cohort should be extended to include these two cytokines, preliminary investigation of the capacity of CBMCs to produce these cytokines was considered. The same stimuli (PHA, beads and SEB) were used and the capacity of CBMCs and PBMCs to produce IL-17 and IL-22 was compared. As at least two-thirds of CBMC samples were expected to produce IL-10 (from Table 4.1), IL-10 production was employed as a control. The response by CBMCs for IL-17 and IL-22 for all stimuli was statistically significantly lower than that for PBMCs (Figure 4.7). For IL-10, the response by CMBCs was significantly reduced for anti-CD3/CD28 beads ($p = 0.0008$) and SEB ($p = 0.0024$) but not for PHA. Because of the very low levels of IL-17 produced the analysis of IL-17 was not extended to the PROBAT cohort.

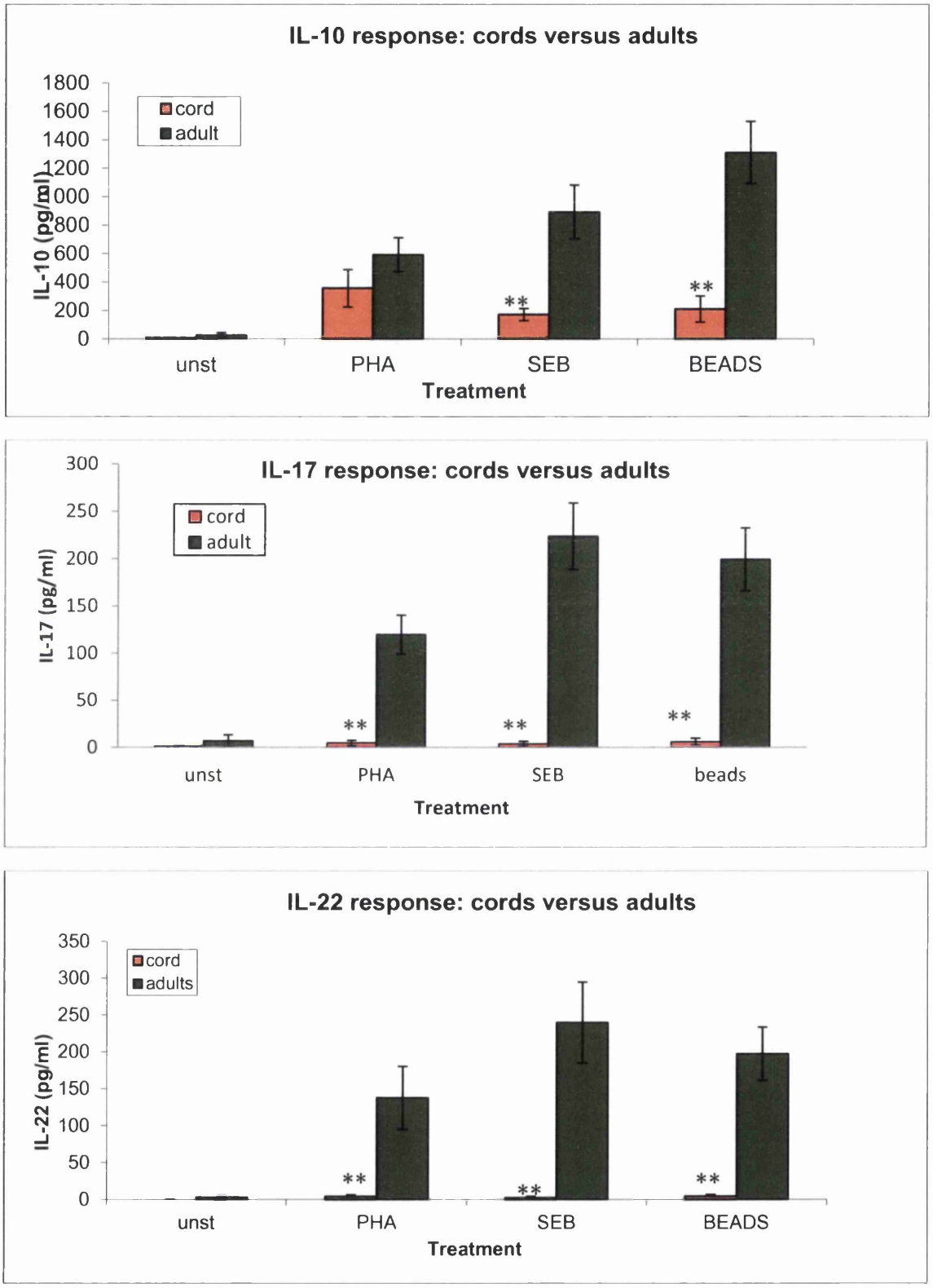


Figure 5.7 Production of IL-10, IL-17 and IL-22 in response to various stimuli.

CBMCs (n = 8 – 10) and PBMCs (n = 9) were left unstimulated (unst) or stimulated with phytohaemagglutinin (PHA), staphylococcal enterotoxin B (SEB) or anti-CD3/CD238 T cell expansion beads (αCD3/CD28) for 48hr. Cell free supernatants were harvested and levels of (a) IL-10, (b) IL-17, and (c) IL-22 (± SEM) measured using specific ELISAs. The response by CBMCs was significantly lower than PBMCs ** $p < 0.01$.

5.4 Discussion

Despite promise in the application of perinatal probiotic supplementation as an intervention strategy to protect against IgE mediated atopy, optimal timings for supplementation and immunological mechanisms underlying protective effects are unresolved. In particular it is not known whether such treatments work (prenatally) or whether postnatal exposure to probiotic bacteria is more important (Boyle *et al.* 2008). The focal point of chapter 5 was to therefore analyse whether perinatal probiotic supplementation had a positive effect on early newborn immune function, in a way that might prevent or attenuate the risk of allergic responses in later life. Before embarking on this experimental rationale, age-related changes in the polyclonal induction of key cytokines involved in the pathophysiology of allergy, by CBMCs or 6 month PBMCs from recruits on the PROBAT trial cohort was first determined. The principal finding emerging from this part of the *ex vivo* study was that there was no statistically significant effect on IL-4, IL-10, IL-13 and IFN-γ production by mitogen stimulated mononuclear cells prepared from the blood of participants on the probiotic and placebo arms of the trial. This finding is discussed in further detail below.

5.4.1 Age related changes in Th2 cytokine induction by CBMCs and 6 month PBMCs prepared from a cohort of potentially atopic subjects in response to the polyclonal stimulation.

5.4.1.1 IL-4

As the cytokine response at birth and in early infancy is generally attenuated compared to that of adults; the proportion of subjects mounting a detectable response for each cytokine and stimuli combination was determined (Table 4.1). The IL-4 response to the stimuli was significantly different between the two age groups for all stimuli studied and there was a statistically significant increase in the proportion of samples returning a detectable cytokine output at 6 months of age compared to birth.

The present study highlighted an age-related maturation of the IL-4 producing T cell compartment from birth to 6 months of age. On comparison of the age related cytokine responses by CBMCs versus 6 month PBMCs prepared from the PROBAT trial birth cohort (Figure 4.1) IL-4 production was higher by 6 month PBMCs samples stimulated with anti-CD3/CD28 beads or SEB. This observation can be

accounted for by the fact that neonatal $CD4^+$ T cells are predominantly of the naive phenotype representing 90% of the circulating T cells population. Since naïve $CD4^+$ T cells have a reduced capacity for cytokine production compared to memory T cells (Clement *et al.* 1988; Clement *et al.* 1990; Janeway 1992), it is plausible that the observed IL-4 increase could be attributable to an increase in the neonatal memory T cell pool at 6 months of age as the newborn matures under antigen experience. Furthermore, decreased cytokine release following *ex vivo* polyclonal stimulation of CBMCs may be reflective of a relative deficiency in post-natal memory T cells in early childhood.

Consistent with the current results Hartel *et al.* (2005) demonstrated that IL-4 mRNA expression levels increase with age in early infancy after PMA/ionomycin stimulation. These results were confirmed in the present study by extracellular detection of IL-4 protein by cytometric bead assay which also revealed an age related correlation of IL-4 protein response. In contrast to the present study, previous reports by Chipeta *et al.* (1998) and Gasparoni *et al.* (2003) noted a low-level intracellular IL-4 expression remaining unperturbed during infancy and childhood. Interestingly, in atopic subjects an expansion of IL-4-secreting cells is correlated to a progressive increase in serum IgE levels and there is a correlation between serum IgE levels and polyclonal IL-4 release in childhood (Tang *et al.* 1993). Further work is, however, necessary to delineate whether differences in the extent of IL-4 production with age can be attributed to age exclusively, or the expression of the early atopic phenotype. Additionally, the effect of probiotic supplementation on regulating the kinetics of IL-4 expression in atopy predisposed individuals still requires further characterisation.

Interestingly, PHA-induced IL-4 secretion has been demonstrated to increased only in highly atopic children and not in those with a lesser degree of atopy (Tang *et al.* 1993). The current study revealed no significant differences in PHA-induced IL-4 production at birth and 6 months of age and contrasts with previous findings on the ontogeny of IL-4 production demonstrating that PHA-induced IL-4 production in healthy non-atopic children increased progressively throughout childhood until adult life (Tang and Kemp 1995).

It has been widely observed that when examining children for IFN-α, IL-4 and IL-5 cytokines, particularly in the 0–2-year age-group, baseline up-regulation of these

cytokines is strongly associated with age (Smart and Kemp 2001). Thus in our study where possible there was a necessity for age-matched controls. For this reason, the IL-4 response was considered in more detail to ascertain whether the same pattern of responders/non-responders was observed in matched cord/infant samples from the same subject at the two different age points. For the response to beads (Figure 4.2b), there was a clear indication IL-4 levels increased between birth and 6 months of age (n = 13). Since IL-4 is produced predominantly by the CD45RO memory T cell repertoire, current results were consistent with the widely accepted dogma that acquisition of infantile memory T cells increases with age, reflecting maturation of T cell immunity in the newborn linked to global increases in cytokine production by infantile mononuclear cells; seen from birth to 6 months in the present study.

5.4.1.2 IL-13

For IL-13, the only significant difference was seen for PHA with the response by CBMCs greater than that by 6 month PBMCs. These results were confirmed by the observations that there were fewer responsive infants for IL-13 for all stimuli. Since neonatal T cells are predominantly naïve $CD45RA^+$, and IL-13 is produced by both activated $CD45RA^+$ naive T cells and $CD45RO^+$ memory T cells, the likely source of *ex vivo* PHA induced IL-13 production by CBMCs would emerge from the naïve CD45RA naive T cell population.

IL-13 is chiefly required by the fetus to maintain the pre-term *in utero* environment, which is immunologically Th2 skewed to prevent fetal rejection (Lin *et al.* 1993; Wegmann *et al.* 1993; Williams *et al.* 2000). Th2 immune responses antagonize Th1 immune responses, which are toxic to placental structural integrity. Furthermore, intrinsic maternal regulatory T cell mechanisms limiting Th1 responses may also be transferred to the fetus via the umbilical cord (Leber *et al.* 2010). This provides a logical explanation as to why post-natal PHA induced IL-13 responses by CBMCs were significantly greater than that by 6 month infant PBMCs. Conversely, lower infant PHA induced IL-13 responses concomitant with fewer responsive infants for IL-13 for all mitogenic stimuli employed can be in part explained by the accepted dogma that upon maturation of newborn immunity there is a move to enhance IgA responses for normal mucosal immunity with age as the developing newborn is introduced to a diverse variety of highly antigenic foods. IgA provides antibody mediated immunity towards food antigens as opposed to IgE up-regulated in atopic

individuals to propagate the allergic response. Interestingly, in contrast to the present study Williams *et al.* (2000) demonstrated that polyclonally induced IL-13 production by CBMCs did not differ from polyclonally induced IL-13 production by PBMCs. In the context of the expression of a clinical atopic disease, Prescott *et al.* (2008) observed that attenuated neonatal PHA induced cytokine responses were associated with atopic dermatitis along with elevated allergen-specific IL-13 at 6 months associated with atopic dermatitis at 2.5 years. In the context of the study of Allen *et al.* (2014) who utilized a cohort of predominantly atopic predisposed individuals, it is of interest that the PHA induced *ex vivo* IL-13 response showed an inverse association with age, suggesting a reduction in a key allergy exacerbating cytokine in the PROBAT cohort with age. This is an interesting find when considered in the context of the secondary outcomes of the overarching trial and the role IL-13 plays in promoting IgE class switching, inhibiting inflammatory cytokine production and increasing the propensity of an individual towards an IgE sensitised state (Wynn 2003).

5.4.1.3 IL-10

IL-10 is a crucial anti-inflammatory cytokine relevant for T-cell regulation and suppression of the T_h1 cytokines, and several IL-10 single nucleotide polymorphisms exist in atopic disease settings (Raedler *et al.* 2012) . IL-10 mediated immune responses are postulated to mature with age upon antigen experience, to maintain immune tolerance (Hadis *et al.* 2011; Renz *et al.* 2011). We therefore hypothesised that in response to the mitogenic stimuli the IL-10 response would increase with age. As hypothesised, the IL-10 bead induced T cell response was greater in the infants, but unexpectedly the PHA-induced response was greater by CBMCs.

5.4.1.4 IFN-γ

Considering newborns recruited on the PROBAT trial were predominantly of an atopic status, it was unexpected that no differences for IFN-γ production by CBMCs or 6 month PBMCs in responses to any of the stimuli were observed. There were fewer responsive infants for SEB-stimulated IFN-γ. Numerous studies demonstrate that the IFN- γ response to polyclonal stimuli is reduced in childhood atopic disorder. Paradoxically, some atopic children present with an increase in IFN- γ secretion throughout childhood (Tang *et al.* 1993; Tang and Kemp 1994; Tang *et al.* 1995;

Campbell and Kemp 1997; Nurse *et al.* 1997; Campbell *et al.* 1998). Since resolution of the symptoms of atopic dermatitis and food allergy occur later on in life (Smart and Kemp 2001); it is plausible that analysis of the mononuclear cell, mitogen induced, IFN-γ response at a later age-point may correspond to an increase in IFN-γ with age. This notion is supported by the observation that in adults, <50– 60% of circulating T cells are of the CD45RO memory-cell phenotype, and memory T cells have been demonstrated to produce <10-fold more IFN-γ than naive T cells (Wilson 1991).

In summary, age-related changes in IL-4, IL-13, and IL-10 production highlighted in this study may reflect physiological maturation of the immune repertoire as maternal antibodies diminish within the first 6 months of life, and *de novo* synthesis of immunoglobulins becomes essential. Current results indicate that this is likely to be mediated in an IL-4 dependent manner and possibly to a lesser extent IL-13 since as ex vivo IL-4 production showed the strongest correlation with age. IL-4 and IL-13 are regarded as key drivers for the induction of Th2 responses (Seder and Paul 1994) necessary for promoting humoral immune responses and maturation of adaptive immune responses in the newborn (Hartel *et al.* 2005). Current results were consistent with the findings of other birth cohort allergic disease prevention studies and reinforce observations by numerous authors highlighting the presence of an evolutionary conserved mechanism in the neonate to limit Th1 responses from birth to early infancy even if the resulting immune equilibrium potentially encourages an immunological arena favourable for the development of allergic responses. Therefore avoidance of the up-regulation of the Th1 inflammatory responses during infancy may be vital survival mechanism of the newborn.

5.4.1.5 IL-17 and IL-22 production in early infancy

Since the publication of perinatal supplementation studies, interest in the contribution of IL-17 to atopy and atopic disease has emerged. Initial investigations as part of this thesis of the capacity of newborns to produce IL-17 and IL-22 produced preferentially by Th17 cells found that similarly to other cytokines the production of these is attenuated in newborns versus adults. Therefore, the cytokine analysis for the PROBAT study was not extended to IL-17. This might be an oversight as differences might emerge that relate to probiotic versus placebo supplemented study participants.

Since culture supernatants are still available analysis of IL-17 or other cytokines could be considered once the clinical effect of the study consortium is determined.

5.5 Cytokine responses by probiotic versus placebo

The present study examined the effects of probiotic supplementation on *ex vivo* cytokine responses by CBMCs and 6 month PBMC cultures stimulated with the polyclonal stimuli (PHA, SEB or anti-CD3/CD28 beads). There were no statistically significant differences between the placebo and probiotic group. The current results are in line with studies conducted by numerous authors documenting the effects of perinatal probiotic supplementation on maturation of immune function in potentially atopic newborns. In these studies newborns preferentially selected for probiotic prevention studies were those born into families harbouring an increased genetic risk of atopy (i.e. ≥ 1 family member with atopic disease). Studies assessing specific effects of probiotic supplementation on *ex vivo* immune responses from newborn hematopoietic cells and those studies adopting a similar perinatal supplementation strategy to the current study will be briefly discussed.

Since IL-4 and IL-13 are drivers of IgE antibody induction and the results of the current study revealed no significant difference in the levels of these cytokines within the probiotic and placebo groups it is reasonable to hypothesis that these results may provide insight into the clinical outcomes of the PROBAT trial and imply no significant differences in IgE mediated atopic sensitization between the placebo and probiotic groups. This hypothesis is in line with observations made by Kalliomaki *et al.* (2001) who administered 1 x 10^{10} *Lactobacillus* GG (LGG) CFU daily to lactating mothers 2 – 4 weeks before delivery and continued supplementation to their newborns until 6 months of age. Despite a statistically significant 50% reduction in the occurrence of atopic eczema occurring in the probiotic group compared to the placebo group, there was no recognizable immunological effect on atopy, no differences in the level of total IgE, or frequency of those with high total IgE or frequency of increased antigen-specific IgE and positive skin prick test results at 3, 12 and 24 month age-points.

Further supporting the results of the current study Kopp *et al.* (2008) randomly assigned pregnant women with at least one first-degree relative or a partner with an atopic disease to receive either the probiotic LGG at 5 x 10^9 colony-forming units twice daily or placebo 4-6 weeks before expected delivery. Following a post-natal period of 6 months, ex *vivo* cytokine responses by newborn mononuclear cells CBMCs (n=68) from neonates taking probiotic or placebo exposed to either IL-2, β-lactoglobulin or inactivated (70% ethanol) it was demonstrated that although LGG induced release of IL-10 and IFN-γ by CBMCs no differences between the study groups for either LGG- or PHA-stimulated cytokine responses were observed. Interestingly, these results were in contrast to their previous studies, indicating that *in vitro* stimulation with LGG resulted in significantly enhanced release of IL-10 and IFN-α by CBMCs, compared with cytokine release in un-stimulated controls. This suggested that the immunomodulatory potential of probiotic bacteria to preferentially regulate key cytokines of the Th1/Th2 immune axis in favour of an anti-allergy response *in vitro* does not necessarily translate to similar immunomodulatory effects *in vivo* in atopically predisposed individuals.

In a randomized double-blind study, Marschan *et al.* (2008) administered probiotic bacteria or placebo for 1 month before delivery to mothers, and for 6 months to their infants with a high genetic risk of allergy. In contrast to the current study, the effect of supplementation was measured in plasma samples for the presence of key inflammatory protein markers, immunoglobulin expression and influence on cytokines of the Th1/Th2 axis as the principal measurement. Interestingly, infants receiving probiotic bacteria had significantly higher plasma levels of C-reactive protein (P=0.008), total IgA (P=0.016), total IgE (P=0.047), and IL-10 (P=0.002) than infants in the placebo group. This result suggest that *ex vivo* plasma analysis may have served as a useful option to understand underlying immunological responses exhibited by the study consortium *in vivo.* Since plasma samples have been collected this type of analyses could form the basis of future work. Similarly to the results of the current study, probiotic supplementation had no effect on IL-4 and IFN-γ levels albeit at the plasma level. CBMCs from neonates on the same study were also stimulated with (β-LG) and PHA prior to *ex vivo* monitoring of IL-4, IL-5, IL-10, IL-13 and IFN-α levels. Similar to the present study, PHA induced IFN-α, IL-4, IL-10, and IL-13 by CBMCs did not differ between probiotic and placebo groups,

indicating that maternal probiotic supplementation had no effect on the cytokine profile.

Employing a rationale different to the current study, Boyle *et al.*(2008) in a pre-natal supplemental study measured fetal immune responses employing CBMCs prepared from cryopreserved cord blood from the newborns of (n = 73) study participants supplemented with1.8 x 10^{10} *L. rhamnosus* GG CFU daily from 36 weeks of pregnancy. Upon analysis of fetal response to heat-killed LGG no difference in cytokine (IL-10, IL-13, and TNF-α, IFN-γ, TGF-β1, and IL-12p40) was observed. LGG treatment of pregnant women did not influence cytokine secretion in CBMCs cultured with heat-killed LGG.

Employing a similar methodology, West *et al.* (2009) supplemented infant cereals with (n =89) or without (n = 90) *Lactobacillus* F19 (LF19) from 4 to 13 months. At the end of the study PBMCs were obtained and cytokine responses to the T cell mitogens anti-CD3 and anti-CD28 monoclonal antibodies determined. The ratio of IFN-γ to IL-4 mRNA expression levels in polyclonally stimulated peripheral blood T cells from recruits was measured as a proxy for immune balance. Interestingly, the authors discovered that LF19 was associated with elevated IFN-γ/IL-4 mRNA ratio in polyclonally activated T cells at 13 months of age and reduced cumulative incidence of eczema. The higher Th1/Th2 ratio in the probiotic compared with the placebo implicated an effect of LF19 on the T cell-mediated immune response. Suggesting that further analysis of the Th1/Th2 ratio in the probiotic versus placebo group in the PROBAT study may provide further insight into the effects of supplementation with the study consortium.

Reinforcing the results of West *et al.* (2009) in another disease treatment study, 4 weeks supplementation with *Lactobacillus rhamnosus GG* was associated with enhanced IL-10 production by mitogen-stimulated PBMCs isolated from children with AD (Pessi *et al.* 2000) and in a comparable study in which LGG was given to infants and children with cow's milk allergy and IgE-mediated eczema for 4 weeks, enhanced IFN-γ responses to polyclonal T-cell activation was also observed in the probiotic compared with placebo group (Pohjavuori *et al.* 2004). Augmenting these results, eight weeks supplementation with *Lactobacillus fermentum* to very young children with eczema reduced the severity of disease and increased polyclonal IFN-γ

responses by T cells in probiotic-treated compared with placebo-treated children (Prescott *et al.* 2005).

In a study population comprising of infants participating in a clinical trial on the effects of probiotics on symptoms of atopic dermatitis, 132 infants were randomly selected by Marschan (2007) who employed a randomized, double-blind design concomitant with an elimination diet. Infants were under 12 months of age, had eczema, and were suspected to have cow`s milk allergy. *Lactobacillus rhamnosus* GG (ATCC 53103) 5 x 10^9 cfu, or a mixture of four probiotic strains (MIX): LGG 5 x 10^9 cfu, *Lactobacillus rhamnosus* LC705 5 x 10^9 cfu, *Bifidobacterium breve* Bbi99 2 x 10^8 cfu, and *Propionibacterium freudenreichii ssp. shermanii* JS 2 x 10^9 cfu, or placebo was administered for 4 weeks to the infants. Blood samples were collected before and after the treatment and infants and their breast-feeding mothers began a Cow's milk-free diet. Infants were randomized to receive a probiotic (LGG or MIX) or placebo product and followed for up to 12 weeks by the study. Interestingly the authors demonstrated that in infants with Cow's milk allergy, anti-CD28-stimulated IFN- γ secretion of PBMC increased significantly in LGG group ($p = 0.023$) suggesting an *in vivo* up-regulation of Th1 immunity by LGG, but no changes occurred in follow-up samples in the MIX group ($p = 0.239$) or placebo groups ($p = 0.086$). IFN- γ secretions appeared to potentiated more in the LGG group compared to the placebo group ($p = 0.006$), but in the MIX group, the IFN- γ level increase was not significant in comparison with the placebo group ($p = 0.058$). Consistent with the results of the current study no differences were detectable between IL-4 secretion in the LGG and placebo groups. Interestingly in infants with IgE-associated eczema, anti-CD28-stimulated IFN- γ secretion of PBMC was potentiated significantly in follow-up samples in the LGG group ($p = 0.048$), but no changes occurred in the MIX ($p = 1.000$) or placebo groups ($p = 0.158$). In comparison with the placebo, the level of IFN- γ secretion increased in the LGG group ($p = 0.017$), but no differences appeared in the MIX group in comparison with placebo ($p = 0.319$). Interestingly probiotic treatment had no significant effect on IL-4 secretion in infants with IgE-associated eczema. As in the present study probiotic treatment had no effect on ex vivo IL-4 or IFN- γ secretion in stimulated (CD28 antibodies) PBMCs.

The PROBAT trial did not have any effect on *ex vivo* IL-4, IL-10, IL-13 and IFN-γ cytokine responses induced by mitogenic stimulation of mononuclear cells isolated

from the study participants at birth or at 6 months of age. These results were comparable to findings from numerous studies adopting the same or similar rationale employing a probiotic consortium or exclusively administered probiotic strain, to assess the effects of supplementation on atopic disorder.

Interestingly, the results of probiotic treatment studies on neonates already presenting with atopic disorder generally indicate a positive effect on eczema which may occur via a pathway of improved maturation or recovery of Th1 type immune responses in probiotic treated infants (Pohjavuori *et al.* 2004; Prescott *et al.* 2005; West *et al.* 2009). Effects appeared to be greatest when a single probiotic strain was used in the intervention as opposed to a consortium as in the present study.

5.6 Limitations of the study

Evaluation of the effect of perinatal probiotic supplementation on age-related changes has been a focal point of the current study in which the immune response of both newborns and infants has been assessed concurrently. Despite the ontogeny of IL-4 production showing the strongest correlation with age in all reviewed studies, a comprehensive review of age-related changes in immune function has given rise to varied results. Reasons for these discrepancies can be attributed to heterogeneity in experimental conditions e.g. mitogenic stimulus employed, method of mononuclear cell preparation and age point for evaluation. Therefore, robust meta-analyses defining the ontogeny of cytokine production with age is needed so immune responses occurring with age that could potentially skew an immune effect of a probiotic supplementation study can be known beforehand. Additionally comparison of reports on age-related alterations in cytokine production is made more difficult by the various techniques used to quantitate cytokines. Most studies measure cytokines in supernatants employing ELISA or Radioimmunoassay, giving rise to high intra-assay and inter-assay variation. Additionally, measurement of the effects of probiotic supplementation on immunological parameters in the context of *ex vivo* cytokine responses by CBMCs and 6 month PBMCs in responses to mitogenic stimuli are largely experimental as the stimulus employed in the current study are non-specific antigen independent stimuli. This could account for the reason why almost all probiotic intervention studies adopting this rationale have revealed no significant differences between probiotic and placebo groups. Some studies have evaluated cytokine mRNA produced by cells to avoid problems of measuring proteins in the

supernatants of experimentally stimulated cells, to limit false positive results that can occur in artificially induced experimental scenarios in which immune parameters are explored. In the context of the current study it may have been better to ascertain the *ex vivo* effects of perinatal probiotic supplementation on the underlying Th1/Th2 ratio, by extracting the RNA from the PROBAT mononuclear cell samples and conducting gene expression analysis of the relevant mRNA cytokine transcripts. This type of analysis would be independent of prior mitogenic stimulation and obviate variations in immunological responses that can ensue as a result of mitogenic stimulation.

While the levels of mRNA provide useful information regarding age associated alterations in transcription of cytokine genes, mRNA results do not always reflect the amount of protein produced and secreted. It is, therefore, difficult to resolve disparities among studies when different methods of cytokine analyses have been employed. Clearly, the combination of these variations in experimental design could significantly contribute to the varying outcomes, even when all other parameters of the study population are controlled. Interestingly despite differences in experimental design among perinatal probiotic intervention studies the current results are line with all the perinatal probiotic intervention studies demonstrating that there are no significant effects of probiotic supplementation on ex vivo immune responses produced by newborn or early infant mononuclear cells, however there are a paucity of studies to indicate whether this predisposition changes upon maturity beyond the 5 year old age point.

Ethical considerations

The probiotic strains used in the study have well-documented safety properties (Allen *et al.* 2010). Previous studies of probiotic supplementation have demonstrated positive clinical effects on the prevention and treatment of atopic diseases (Majamaa and Isolauri 1997; Isolauri *et al.* 2000; Kalliomäki *et al.* 2001, 2003; Rosenfeldt *et al.* 2003; Viljanen *et al.* 2005; Abrahamsson *et al.* 2007). Based on these findings, we assumed that infants would benefit from probiotic treatment.

5.7 Conclusions

Research employing the application of the polyclonal mitogens has considerably enhanced understanding of immune responses during early infancy and clarified the

mechanisms of allergic diseases and IgE sensitization. Although probiotic bacteria offer an attractive possibility for an immunomodulatory intervention in the treatment of atopic disorder, the underlying immunological mechanism imparted by probiotic bacteria to do so remain unresolved in the current study. Upon analysis of the effects of probiotic supplementation on *ex vivo* IFN-γ, IL-10, IL-13 and IL-4 responses by umbilical cord blood and 6 month infant blood mononuclear cultures stimulated with the polyclonal stimuli (PHA, SEB or anti-CD3/CD28 beads) at each age point. There was no statistically significant immunomodulatory effect of perinatal probiotic supplementation when compared to the placebo. Although interest is growing in developing therapeutic applications and primary prevention strategies for allergic diseases, essential to this approach is a clear understanding of the immunological processes in early life which promote IgE-sensitization leading to allergic disease in terms of age related changes in the ontogeny of cytokine production from the neonatal stage to early infancy.

Chapter 6: *Ex vivo* effects of perinatal probiotic supplementation on the maturation of innate and adaptive immunity

6.1. Introduction

Profound haematopoietic differences in the cellular composition of human blood are apparent in the newborn and older infant. These differences are marked by an evolutionary conserved age related predisposition favouring granulopoesis, (the formation of granulocytes) in the neonate and lymphopoiesis, (the formation of lymphocytes) in the older infant (Christensen *et al.* 1989). It is widely accepted that lymphocyte subpopulations vary during the neonatal period (Comans-Bitter *et al.* 1997) according to the balance of tolerogenic, allergenic and cellular immunity against pathogens. However, mechanisms by which probiotic bacteria might beneficially affect the cellular landscape of immunity in the atopy predisposed neonate are largely unknown. Furthermore, the effects of perinatal probiotic supplementation in preventing the proliferation of granulocyte and lymphocyte subsets pertinent to the expression of the atopic phenotype remained largely unexplored, particularly within a cohort of clinically predisposed atopic neonates. It is hypothesised that hematopoietic myeloid progenitors support recruitment of pro-inflammatory cells to tissue sites involved in the progression of atopic disease

(Gauvreau *et al.* 2009). Elevated eosinophil counts at birth and/or at 3 months of age have been implicated in infantile eczema and early diagnosed aopic eczema in early life (Borres *et al.* 1995; Borres and Bjorksten 2004; Matsumoto *et al.* 2005). The clinical observation that hematopoietic progenitors from the cord blood of high risk infants give rise to elevated eosinophil and basophil progenitor levels is widely accepted. The hypothesis, in Chapter 6 was that the immunomodulatory effects of perinatal probiotic supplementation *in vivo* might occur via microbial stimulation of pattern recognition receptors, to curtail the differentiation of eosinophil and basophil progenitors (Reece *et al.* 2011). Defects in the cellular composition of human peripheral blood have been implicated as early pre-symptomatic markers for the development of atopic disorder. Reduced numbers of circulating regulatory T cells concomitant with increased numbers of circulating eosinophils is frequently cited (Fontenot *et al.* 2003; Godfrey *et al.* 2005; Liu *et al.* 2006). This is one of the first studies to investigate the impact of perinatal probiotic consumption on leukocyte counts at birth and 6 months of age.

Perinatal probiotic supplementation is postulated to stimulate development of the Th1 cell repertoire (Eisenhofer *et al.* 1997; Backhed, Ding *et al.* 2004), mediate immune tolerance, and attenuate the development of Th2 mediated atopic disorder (Eckburg *et al.* 2005). Since the principle site of interaction of probiotic bacteria is the intestinal tract, it may appear unrelated that probiotics might exert a protective systemic effect against the development of atopic disorder at sites distal to the intestinal tract, such as the skin and respiratory tract. Nonetheless, propagation of an intestinal probiotic mediated Th1 immunomodulatory signal is achievable systemically via the interaction of probiotic bacteria with (1) dendritic cells; (2) their transytocis via the intestinal epithelium M cells (Hathaway and Kraehenbuhl 2000); (3) and the re-circulatory nature of intestinal lymphocytes interactive with probiotic bacteria that home to other, mucosal sites and exert the effector function.

6.1.1 Aim of the study

The aim was to ascertain whether immunomodulatory signals provided by perinatal supplementation with the study consortium, altered lymphocyte and granulocyte population dynamics, away from a cellular phenotype pertinent to the inception of atopic disorder. This *ex vivo* study combined a flow cytometric and haematological analysis of neonatal whole blood from recruits on the PROBAT trial. Since automated haematology analysis of whole blood only provides information on total

lymphocyte and granulocyte counts, the additional flow cytometric approach was required to analyse in more detail the effects of probiotic supplementation on key lymphocyte subsets, (T cell subsets, B cell subsets and dendritic cell subsets). We are currently one of the first groups to adopt this type of approach in any perinatal probiotic supplemental study for the prevention of atopy. Adopting this approach, umbilical cord blood samples at birth and peripheral blood at 6 months of age from recruits of the PROBAT trial were haematologically analysed using CellDyn and also immunophenotyped by flow cytometry to determine the percentages of key granulocytic and lymphocytic subpopulations involved in atopic disorder. Since lymphocytes continuously leave the blood, enter non-lymphoid and lymphoid organs and finally return to the blood again it is noteworthy that lymphocytes studied adopting this approach are predominantly recirculating in nature. The effects of perinatal probiotic supplementation on the proliferation of granulocytic (e.g. basophils and eosinophils) and lymphocytic subsets (e.g. gut homing lymphocytes, T regulatory cells and memory T cells) within neonatal whole blood was assessed in the context of the pathogenesis of atopic disorder (See Chapter 1, 1.3.4 to 1.3.5) in supplemented neonates.

A principal aim in any perinatal probiotic intervention is to manage the inherent Th2-mediated atopic predisposition in the presence of probiotic organisms capable of inducing Th1 polarising cytokines that challenge the atopic predisposition (Furrie 2005). To monitor this, *ex vivo* whole-blood cultures were established from birth to 6 months of age from recruits on the PROBAT trial and stimulated with lipopolysaccharide (LPS) or peptidoglycan (PGN) which are ubiquitously occurring MAMP encountered by the newborn in the post utero environment, and act here as a measure of innate immune function. The application of the whole blood approach allowed for the contribution from multiple cell types (e.g. neutrophils, dendritic cells and monocytes) as well as soluble factors occurring in plasma (e.g. soluble CD14, LPS binding protein) during microbial induction of the cytokine response. The application of LPS in this study was particularly useful since the allergen Der p 2 functions as a ligand for TLR4, due to its structural homology to the mammalian TLR4 co-receptor MD-2 (Botos *et al.* 2011). These microbial stimulants served as a surrogate measure of granulocyte mediated innate immune function and allowed age related change in cytokine production to be measured *ex vivo* as a consequence of perinatal probiotic supplementation. The cytokines measured were TNF-α and IL-

12p70, serving as a proxy for the Th1 pro-inflammatory response and IL-10 serving as a proxy for the regulatory response. We therefore aimed to measure any underlying changes in the maturation of the innate cytokine immune response as a result of perinatal probiotic supplementation. We hypothesized that supplementation might augment granulocytic responses to microbial stimuli in a Th1 dependent manner in the probiotic group compared to the placebo group.

Due to the ability of the study consortium to modulate induction of Th1 inflammatory cytokines antagonistic to the expression of atopy supporting Th2 cytokines both *in vitro, we* hypothesize that there is a beneficial immunomodulatory role of perinatal probiotic supplementation occurring at the cellular immune level. We postulate that supplementation might contribute to the maturation of regulatory lymphocyte subsets necessary to control the maturation of Th2 dominated cellular immune responses that support the development of atopy in predisposed neonates. We also hypothesised that perinatal probiotic supplementation would accelerate the age related maturation of neonatal Th1 immune responses at the cytokine level and reduce the circulating whole blood eosinophil and basophil counts in the probiotic group compared to the placebo group. We anticipate that the net clinical consequence of these responses would be a resistance of the atopic phenotype in consortium supplemented neonates.

6.2. Materials and Methods

6.2.1 Sample collection

All subjects were participants on the PROBAT trial. Umbilical cord blood was collected from the newborn immediately after birth by a qualified midwife and peripheral infant blood at 6 months of age. Blood collected into sodium heparin (Vacutainer, Greiner Bio-One); gel and clot activator tubes (Vacutainer) were used for all analyses presented in this chapter.

6.2.2 Automated haematology analysis

Full blood counts and differentials were generated using an automated haematology analyser (CellDyn Ruby; Abbott Diagnostics Maidenhead, UK). Total white blood cells, leukocyte populations and cytokine production were measured in umbilical cord blood collected at birth and venous blood collected at the 6 month follow-up.

6.2.3 Whole blood cultures

Heparinised blood was diluted 1 in 4 in RPMI 1640/Glutamax (Invitrogen, Paisley, UK) in a total volume of 600 µl in culture tubes. Whole blood cultures were left untreated or treated with 10ng/ml LPS and 3µg/ml PGN (Invivogen, California, USA). For IL-12p70, cultures were pre-treated for 90 minutes with 10 ng/ml; IFN-γ (PeproTech EC Ltd, London, UK) prior to addition of LPS. After 24 hours incubation at 37°C in 5% CO_2-in-air cell free, culture supernatants were harvested by centrifugation. These were stored at -80°C until batch analysis of cytokines using specific ELISAs in accordance with the manufacturer's instructions (TNFα, IL-10, IL-12p70; OptEIA, BD Biosciences, Oxford, UK).

6.2.4 Flow cytometry - staining

Antibodies were added at predetermined concentrations to FACS tubes and 50µl of whole blood added to each tube. Each tube was vortexed and incubated on ice for 30 minutes in the dark. After this time 3 ml of FACS lyse (BD Biosciences) was added to each tube (except for tube 16 to which 3 mls of Easy Lyse (Dako) was added), the tubes were vortexed and then incubated in the dark at room temperature for a further 10 minutes except for tube 16 which was incubated for 15 minutes. The cells were washed by centrifugation. The supernatant was discarded and the pellets were re-suspended in FACS buffer prior to a further wash by centrifugation. After discarding the supernatant, the cells were re-suspended in 200 µl FACSFIX (BD Biosciences). Tubes were kept in the fridge and acquired on the flow cytometer (FACSAria; BD Biosciences) within 24 hours.

Difficulty arising in obtaining accurate absolute counts of lymphocytes subpopulations by flow cytometric immunophenotyping of neonatal cord blood is caused by erythroid cell contamination of the archetypal lymphocyte gate (Loken *et al.* 1990). This was avoided by application of the reagent Easy Lyse which provides complete and gentle lysis of erythrocytes prior to FACs staining of whole blood.

6.2.5. Statistical analysis

Statistical analysis and p values were obtained to assess the maturation of total wbc, neutrophils lymphocyte, monocytes eosinophils, basophils red blood cells and

platelets and other haematological parameters from birth to 6 months of age. The effect of perinatal probiotic supplementation versus the placebo on hematopoietic ontogeny of the same cell types was determined as mean probiotic difference (as previously described see 5.2.3).

6.3. Results

6.3.1 Cell counts at birth and 6 months of age

As samples were being collected from a large number of newborns (umbilical cord blood n = 100) and 6 month old infants (peripheral blood n = 103) a comparison of changes in counts of various cell populations was undertaken. Automated haematology analysis yields data as total counts and, for leukocytes, percentage of total white blood cell counts. As leukocytes are the main interest of the study the data for these are shown graphically in Figure 6.1 with total counts ($x10^9$/litre; Figure 6.1a) and percentage of total leukocytes (Figure 6.1b).

For cell counts, there were significant differences between birth and 6 months of age for all cell types except red blood cells. Statistically significant higher levels were found in cord blood for total white blood cells, neutrophils, monocytes, eosinophils, basophils and platelets. Only lymphocytes were higher in the infant samples ($p < 0.0001$). Similarly the percentages of each of the leukocyte subsets - neutrophils, monocytes, eosinophils and basophils - were significantly higher in cord blood; only lymphocytes were higher in the infant samples.

Among all the samples analysed there were 45 matched pairs for which data were available for the same infant at birth and 6 months of age (cell counts – Figure 6.2; percentage of total leukocytes – Figure 6.3). These data confirm the dramatic differences in lymphocyte and neutrophil counts/percentages in particular among the subsets studied.

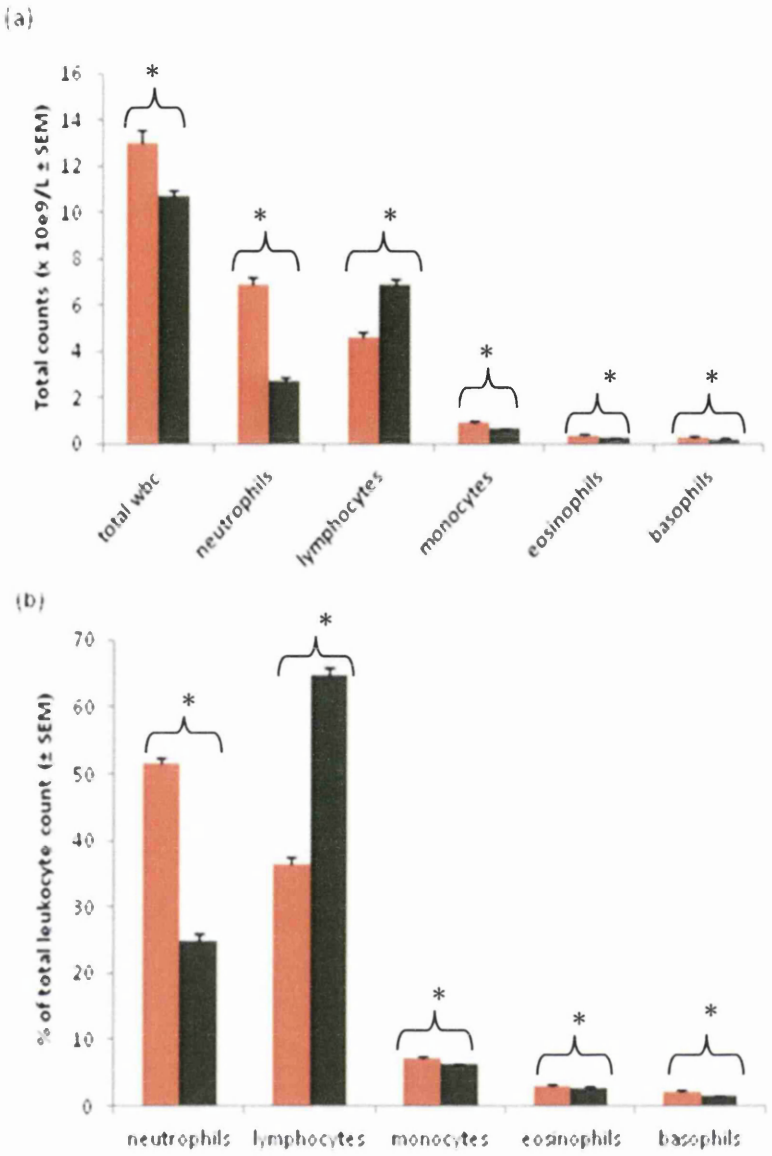


Figure 6.1. Automated haematology analysis of umbilical cord blood (orange) and peripheral blood from 6 month infants (green).
Summary of (a) total counts (x 10^9/L), and percentages of each major leukocyte population in blood collected at birth (n = 100) and at 6 months of age (n = 103). There was a significant difference between the two age groups for all measurements. This was denoted by * $P < 0.01$.Note that percentages are not available (N/A) for total white blood cells or platelets as this is calculated as percentage of total white blood cells

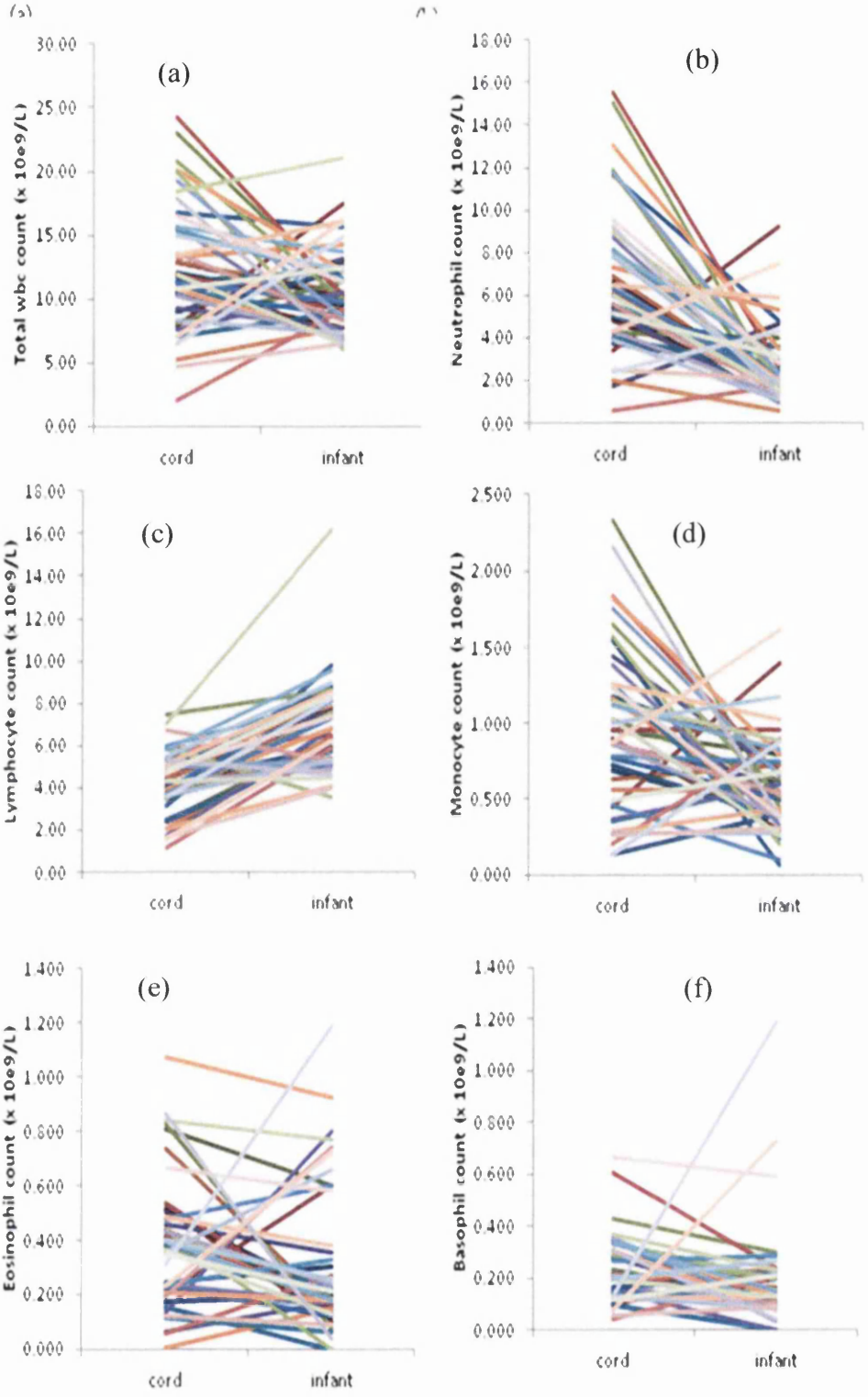


Figure 6.2. nges in total and major leukocyte subset counts with age. Automated haematology analyses was used to generate counts (x 10e9/L) of (a) total white blood cells, (b) neutrophils, (c) lymphocytes, (d) monocytes, (e) eosinophils, and (f) basophils from matched samples of umbilical cord blood and peripheral blood collected at 6 months of age from participants of PROBAT study (n = 45).

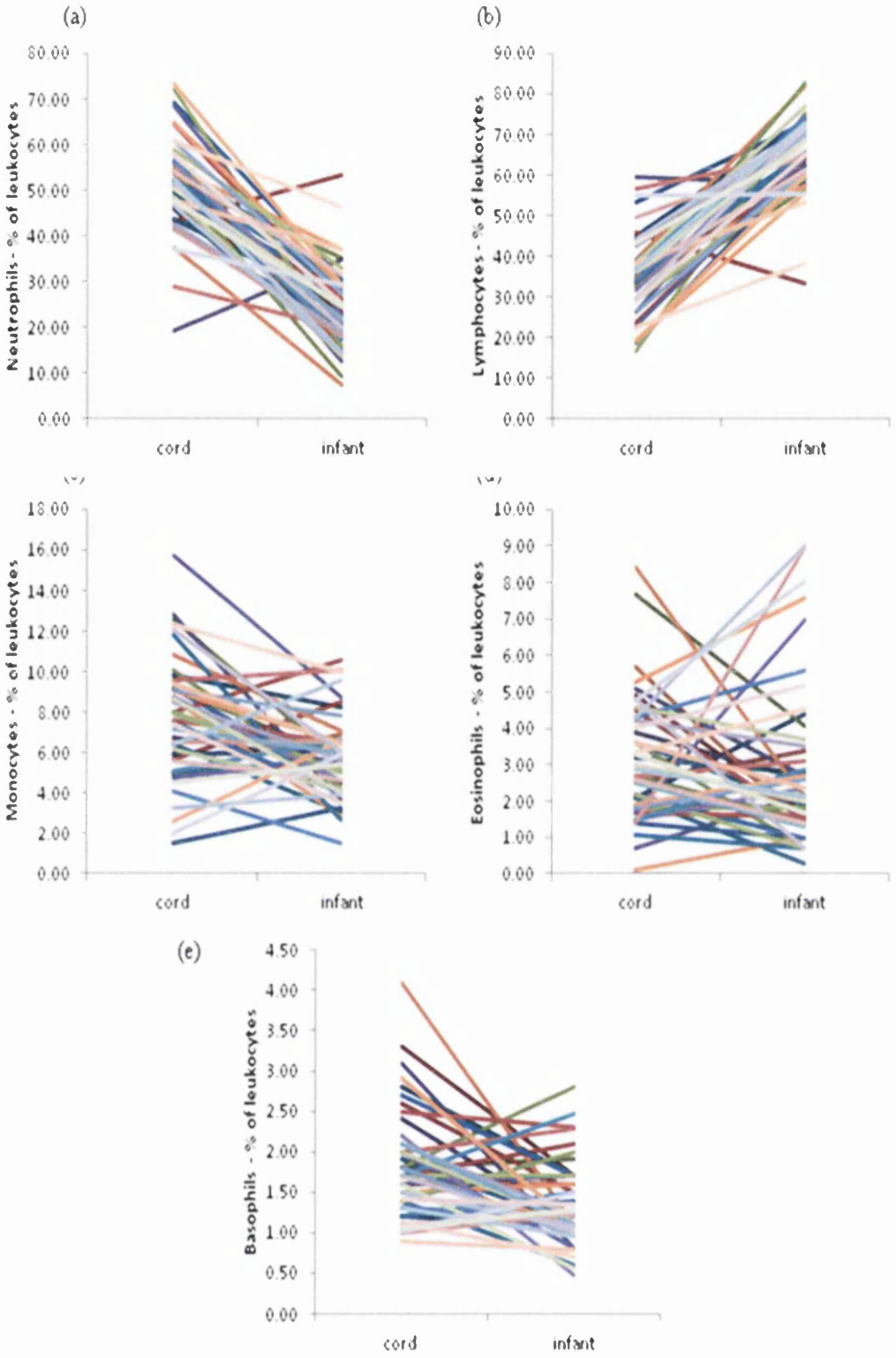


Figure 6.3. hanges in percentages of major leukocyte subsets with age. Automated haematology analyses was used to determine percentage of total leukocytes that were (a) neutrophils, (b) lymphocytes, (c) monocytes, (d) eosinophils, and (e) basophils from matched samples of umbilical cord blood and peripheral blood collected at 6 months of age from participants of PROBAT study (n = 45)

5.3.2 Cell counts in relation to probiotic versus placebo exposure

Counts of total white blood cells, and counts and percentages of leukocyte subsets were compared at birth and 6 months of age for infants recruited to the probiotic or placebo groups of the study (Figures 5.4 – 5.11). The only difference between the two study groups at the two age points was a decreased eosinophil counts at birth in infants whose mothers had taken the probiotic supplement over the last few weeks of pregnancy ($p = 0.0242$). However there were no differences in the percentage of eosinophils at birth or the total counts or percentages of eosinophils at 6 months of age. Circulating platelet (Figure 6.10) and red blood cells (Figure 6.11) numbers and all other haematological parameters investigated did not differ with probiotic or placebo usage at any age.

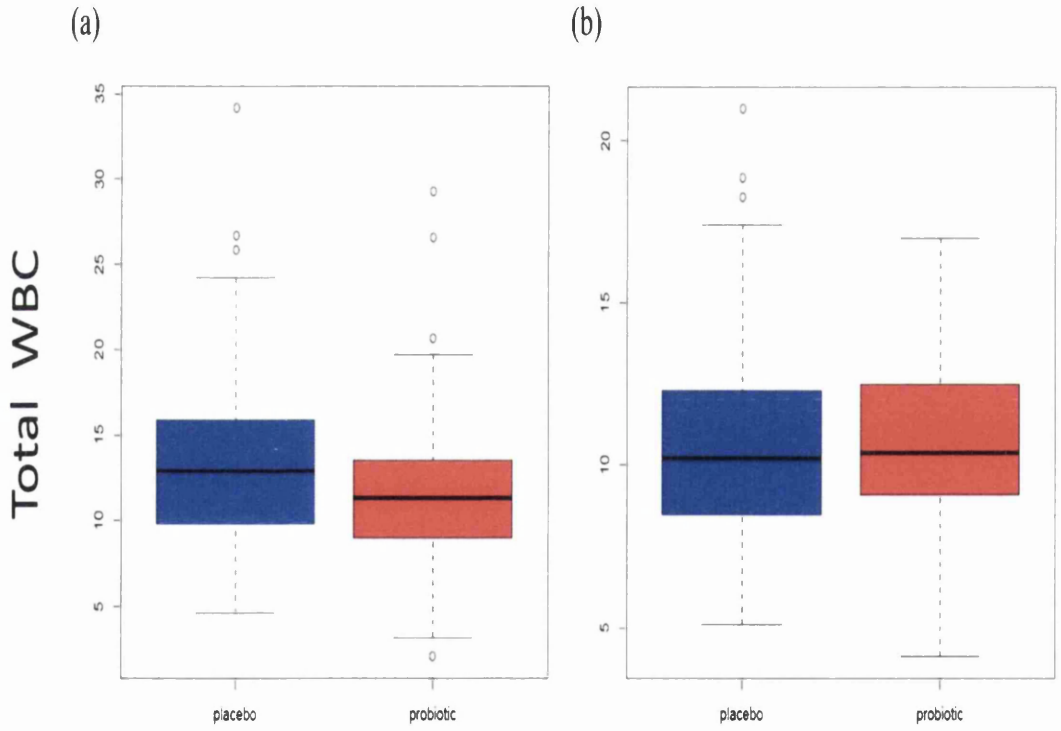


Figure 6.4 Total white blood cell counts from participants in the PROBAT study. Blood was collected at (a) birth (umbilical cord blood), and (b) 6 months of age and used for automated haematology analysis to give total white blood cell counts (x 10^9/litre). The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

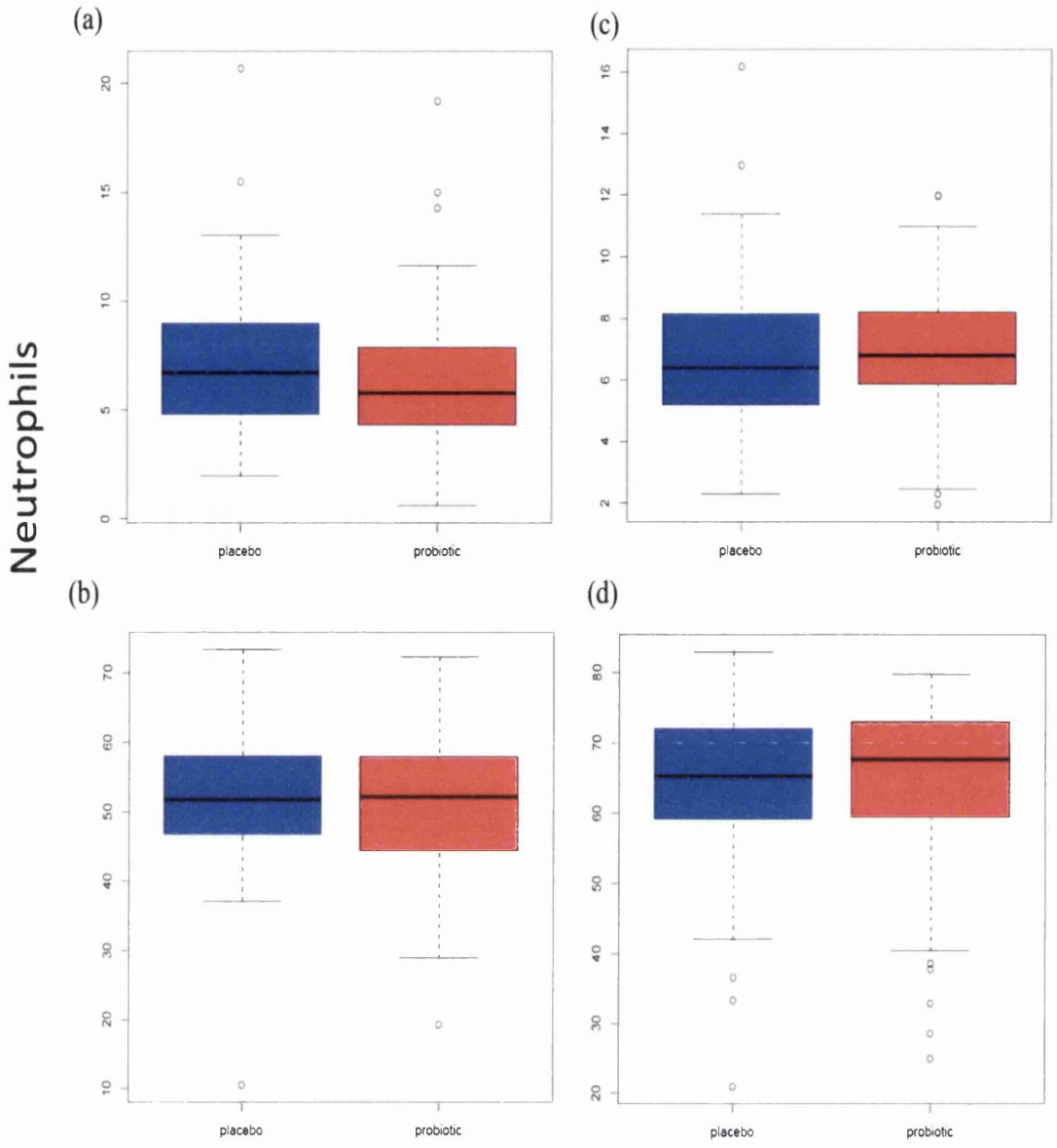


Figure 6.5 Neutrophil counts from participants in the PROBAT study.
Blood was collected at (a & b) birth (umbilical cord blood), and (c & d) 6 months of age, and used for automated haematology analysis to give (a & c) total neutrophil counts (x 10^9/litre), and (b & d) percentage of neutrophils. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

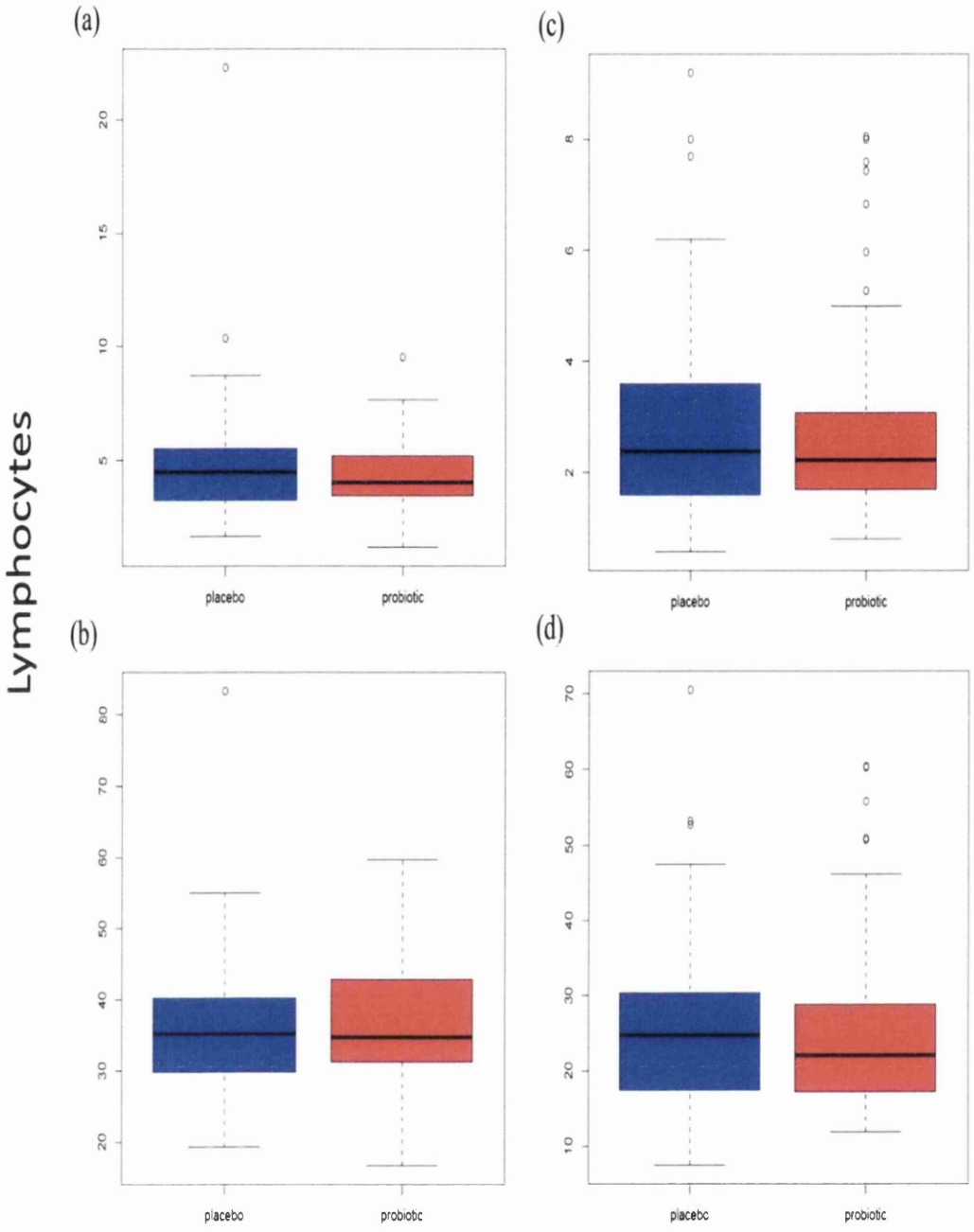


Figure 6.6 Lymphocyte counts from participants in the PROBAT study. Blood was collected at (a & b) birth (umbilical cord blood), and (c & d) 6 months of age, and used for automated haematology analysis to give (a & c) total lymphocyte counts (x 10^9/litre), and (b & d) percentage of lymphocytes. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

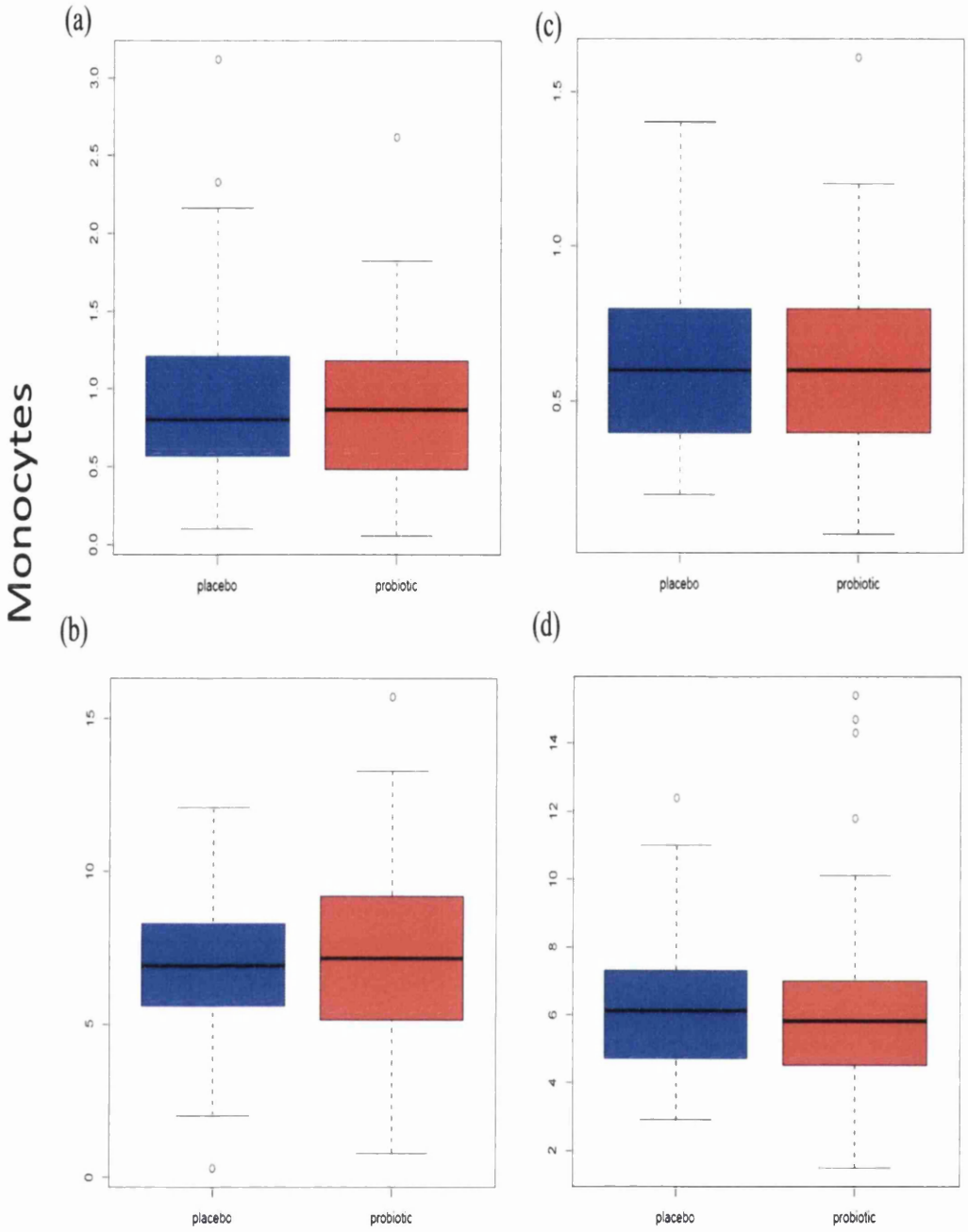


Figure 6.7 Monocyte counts from participants in the PROBAT study.
Blood was collected at (a & b) birth (umbilical cord blood), and (c & d) 6 months of age, and used for automated haematology analysis to give (a & c) monocyte counts (x 10^9/litre), and (b & d) percentage of monocytes. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ○ Statistically significant results ($P < 0.05$) were denoted by *

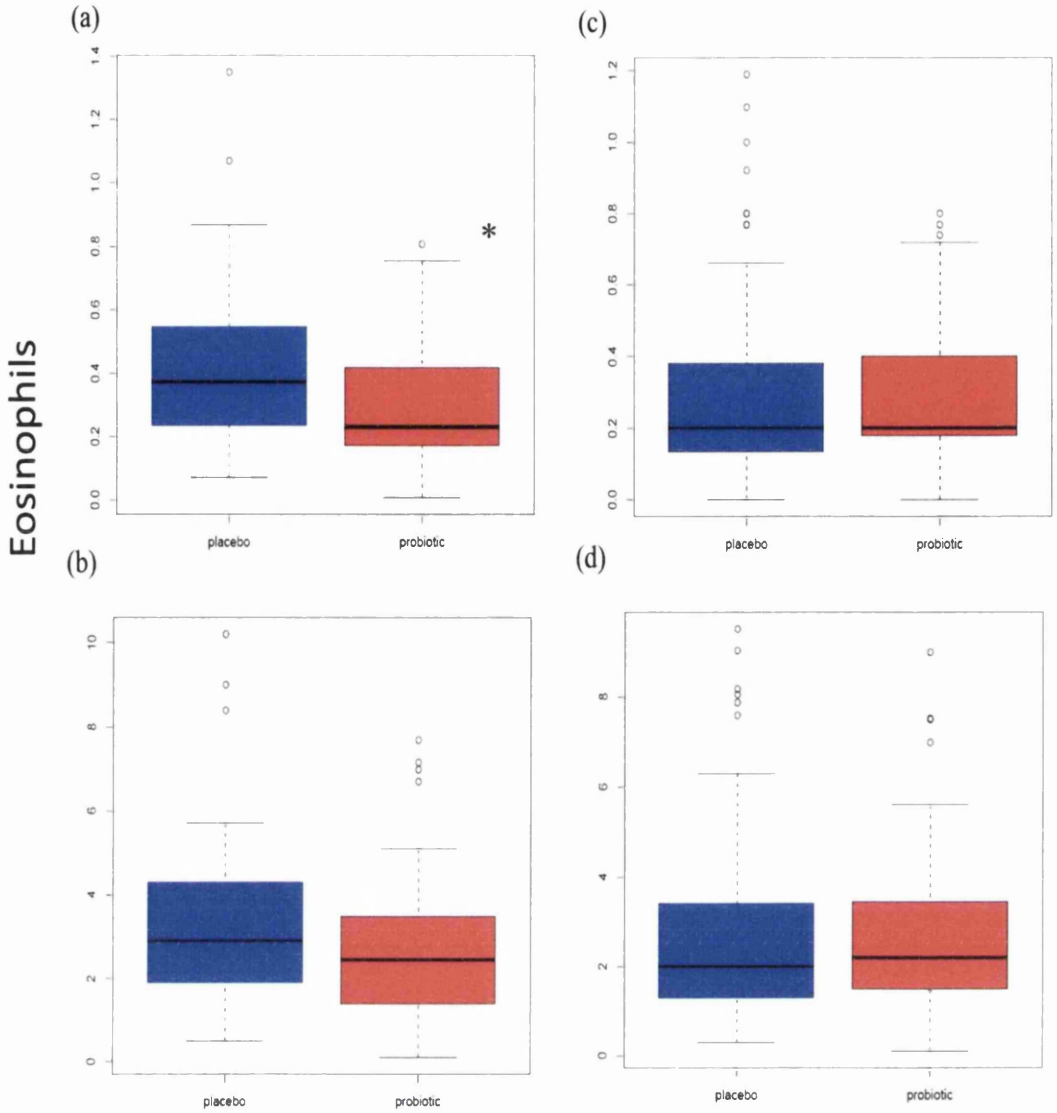


Figure 6.8 Eosinophil counts from participants in the PROBAT study.
Blood was collected at (a & b) birth (umbilical cord blood), and (c & d) 6 months of age, and used for automated haematology analysis to give (a & c) eosinophil counts (x 10^9/litre), and (b & d) percentage of eosinophil. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ○ Statistically significant results ($P < 0.05$) were denoted by *

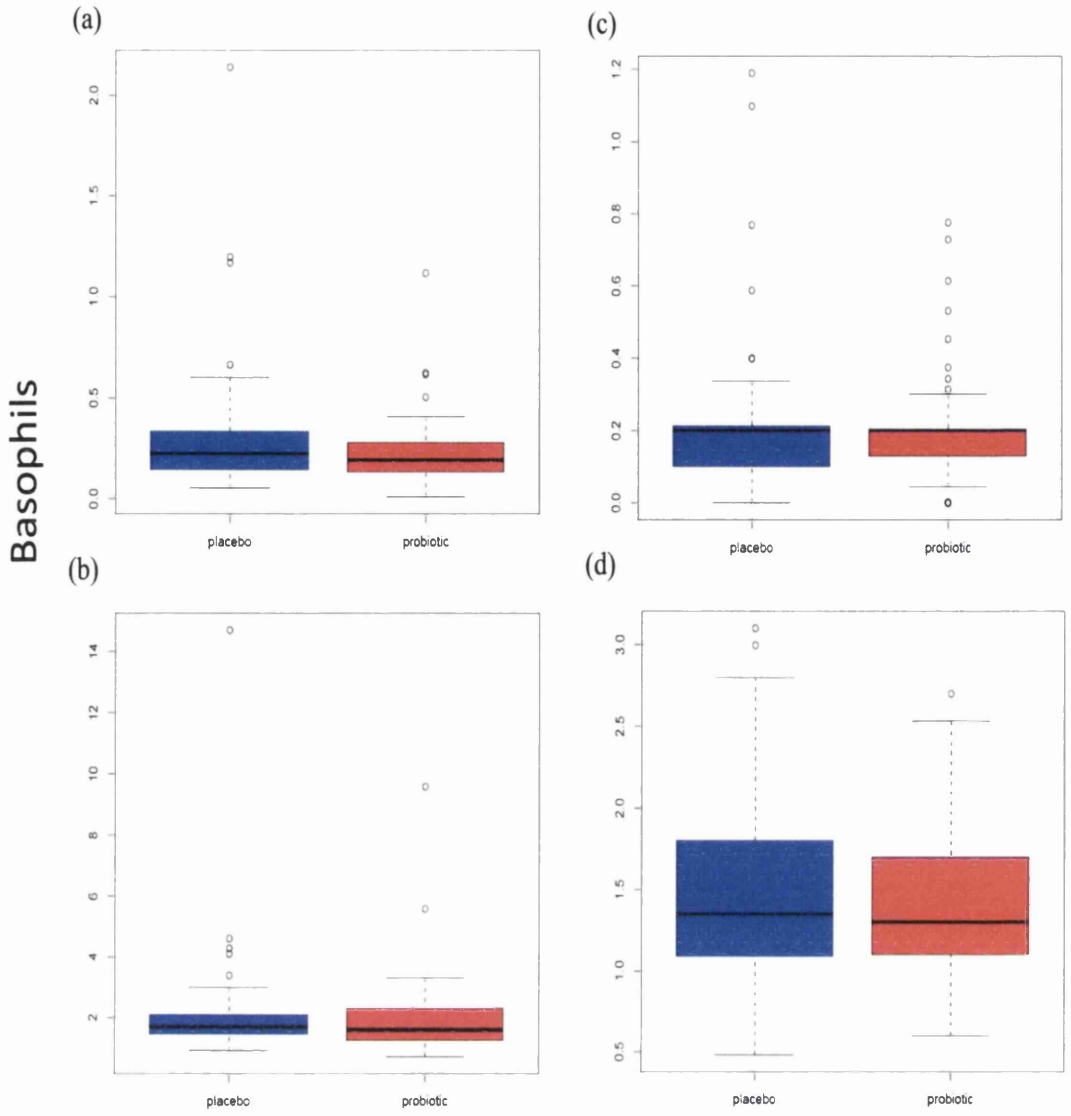


Figure 6.9 Basophil counts from participants in the PROBAT study.
Blood was collected at (a & b) birth (umbilical cord blood), and (c & d) 6 months of age, and used for automated haematology analysis to give (a & c) basophil counts (x 10^9/litre), and (b & d) percentage of basophil. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

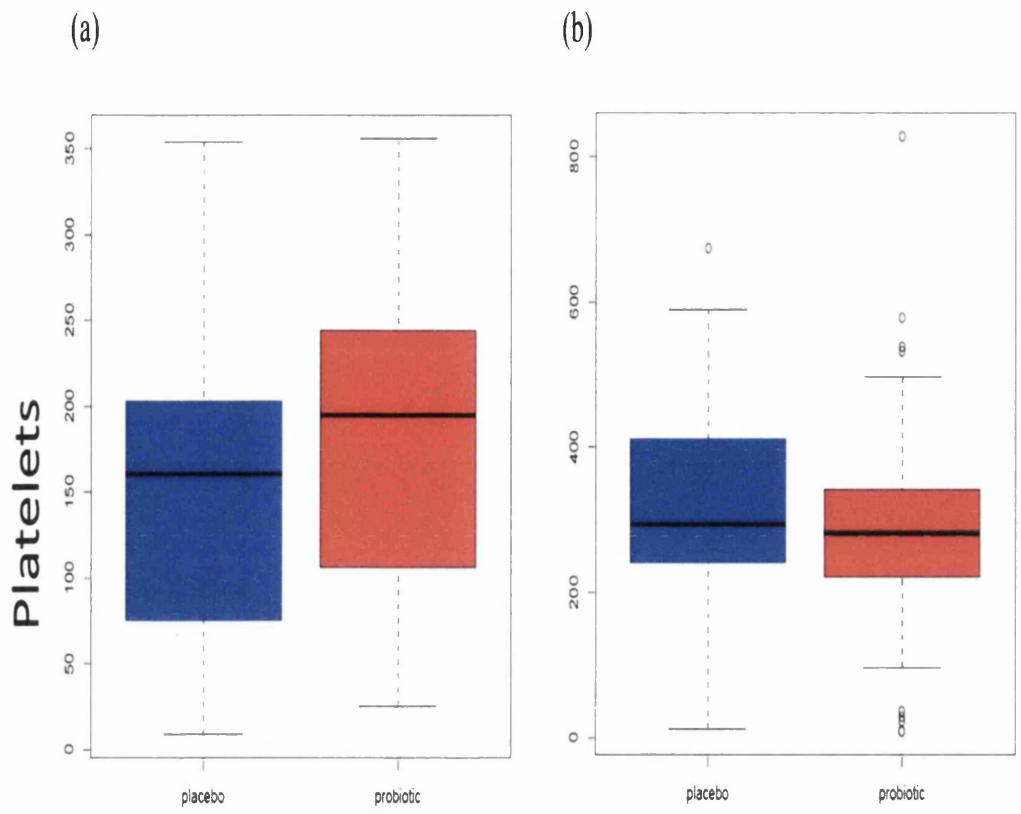


Figure 6.10 Platelet counts from participants in the PROBAT study.
Blood was collected at (a & b) birth (umbilical cord blood), and (c & d) 6 months of age, and used for automated haematology analysis to give (a & c) platelet counts (x 10^9/litre), and (b & d) percentage of platelets. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

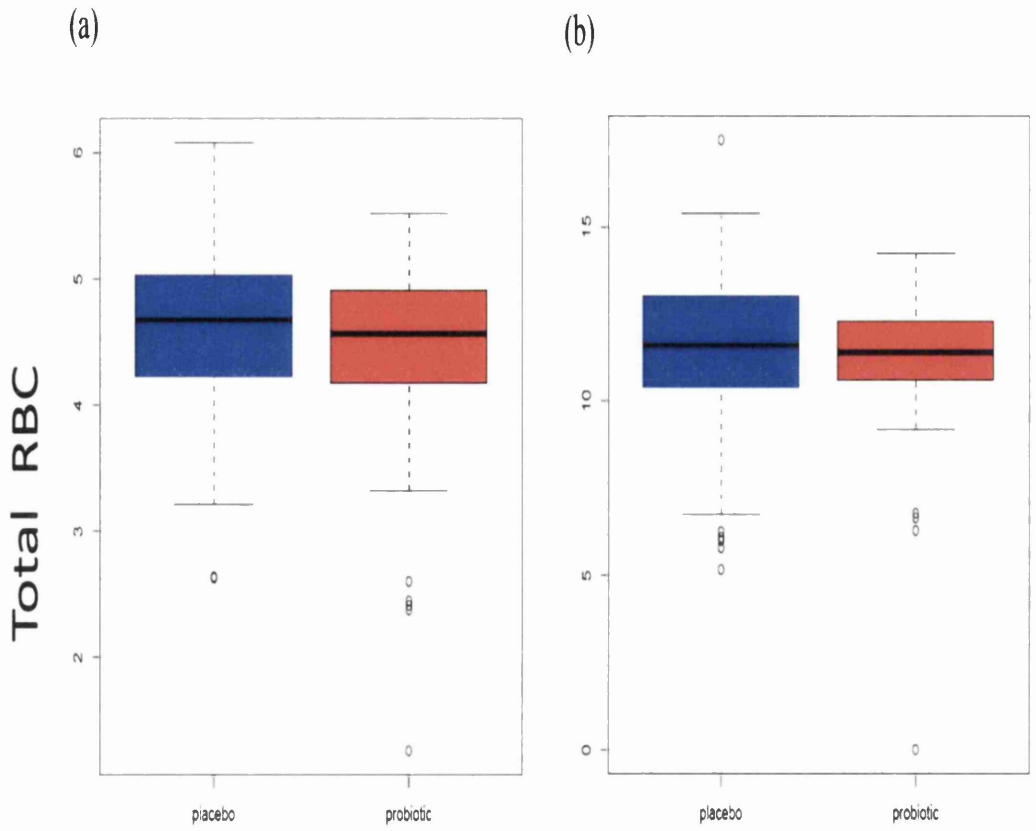


Figure 6.11 Red blood cell counts from participants in the PROBAT study. Blood was collected at (a & b) birth (umbilical cord blood), and (c & d) 6 months of age, and used for automated haematology analysis to give (a & c) red blood cell counts (x 10^9/litre), and (b & d) percentage of red blood cells. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

6.3.3 Innate immune response at birth and 6 months of age

Assessment of age related changes in cytokine responses at birth and 6 months of age, employing the stimuli LPS and PGN, revealed statistically significant increases in IL-10 and TNF-α (Figure 6.12; all $p < 0.0001$) above the background level. For IL-12p70, a statistically significant increase above background was only see for LPS/IFN-γ at birth ($p = 0.032$) and PGN at 6 months of age ($p = 0.005$) (Figure 6.13). The IL-12p70 response to LPS/IFN-γ at 6 months of age was near significant ($p = 0.083$).

Differences in the levels of these cytokines produced in response to LPS or PGN by cord blood versus infant peripheral blood were found: LPS-stimulated IL-10 was significantly higher at 6 months of age than at birth ($p = 0.022$); and PGN-stimulated TNF-α was significantly higher at 6 months of age ($p = 0.013$). As IFN-γ pre-treatment has been identified to maximise IL-12p70 output in response to either LPS or PGN, data for LPS and PGN alone (Figure 6.13a) and with IFN-γ pre-treatment (Figure 6.13b) are shown. IL-12p70 responses were significantly elevated at 6 months of age compared to birth in unstimulated ($p < 0.0001$), PGN ($p < 0.0001$), and unstimulated/IFN-γ ($p < 0.0001$) whole blood cultures.

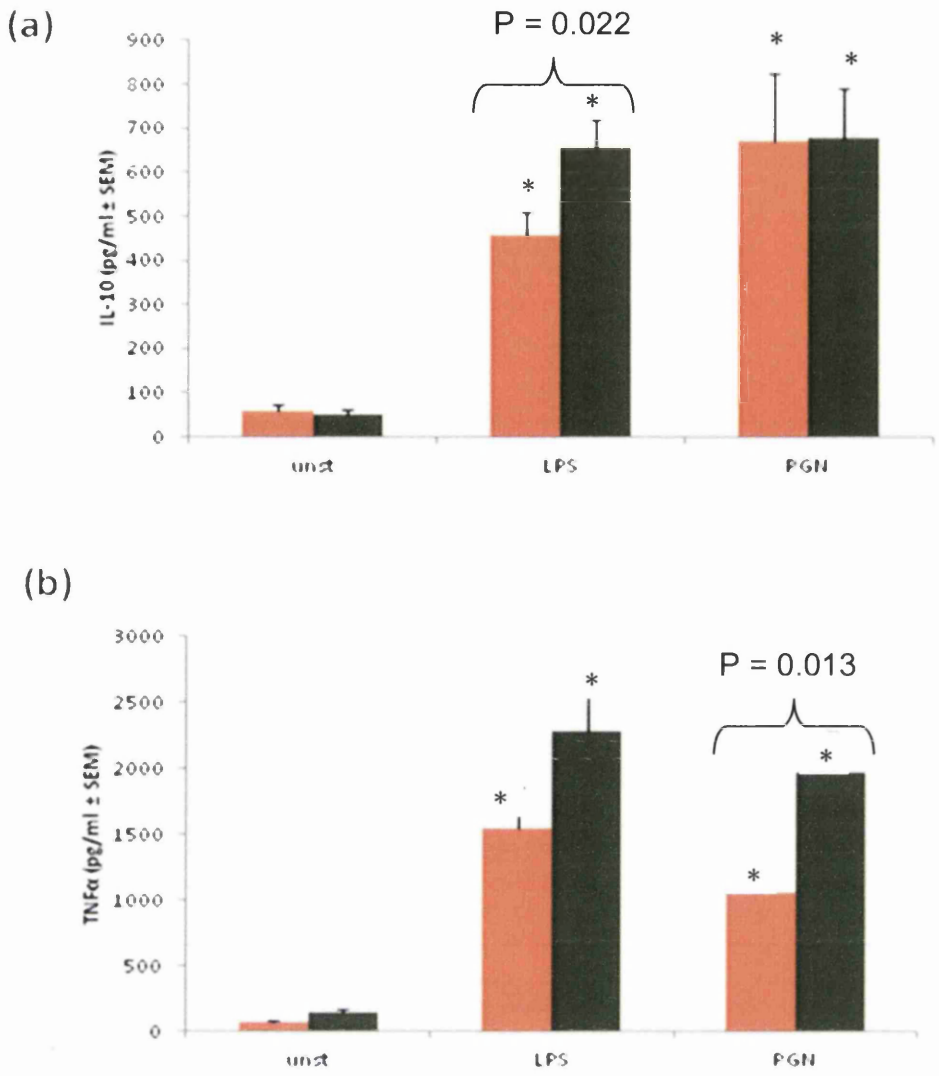


Figure 6.12 IL-10 and TNF-α production by whole blood cultures
Whole blood cultures were prepared from n = 100 umbilical cord blood (orange) and n = 103 peripheral blood of 6 month infants (green), and left unstimulated, or stimulated with LPS or PGN. The mean (a) IL-10 and (b) TNF-α levels ± SEM, in cell free culture supernatants harvested after 24 hour culture were measured using specific ELISA. The student T was used to compare LPS and PGN induced cytokine responses to the background level. P>0.05 was considered statistically significant and denoted with. * The student T was also used to determine whether there were differences between responses from birth to 6 months of age, statistically significant differences (P>0.05) are indicated on the graph above the bracket.

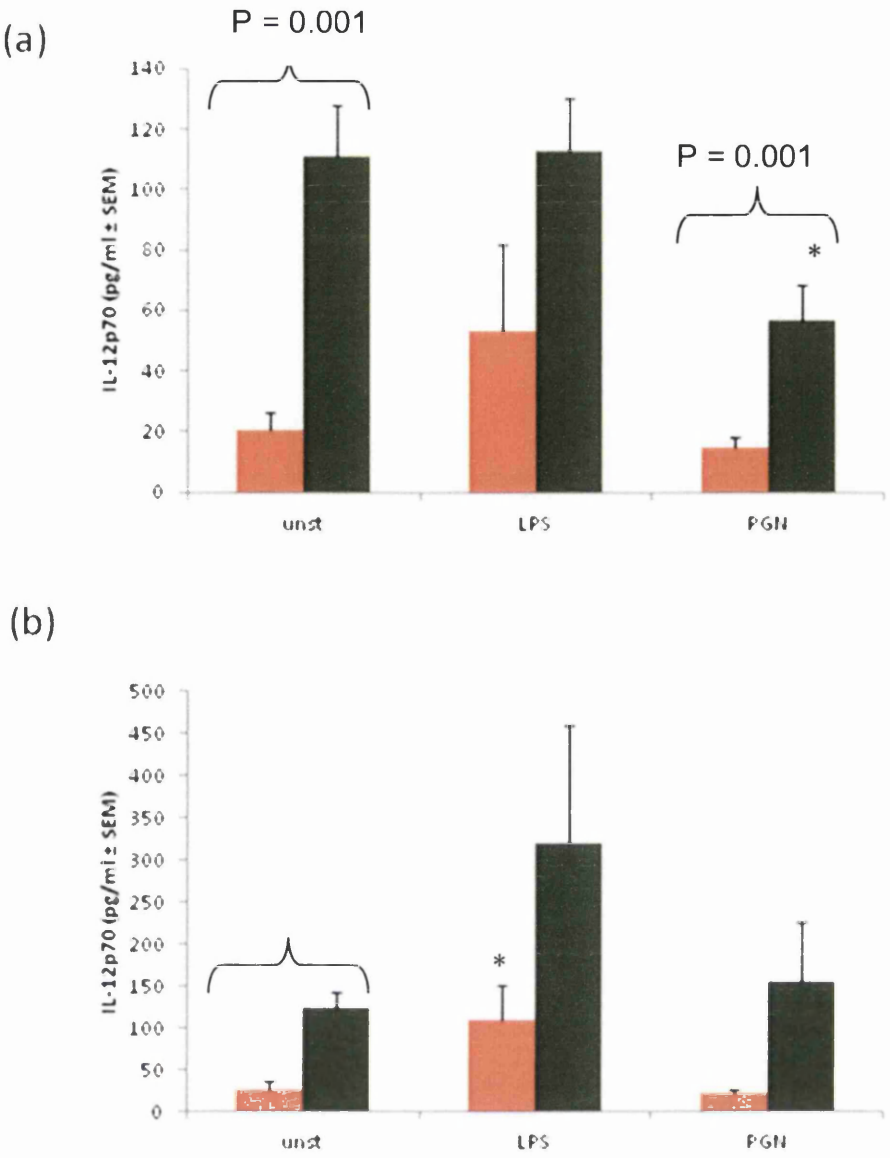


Figure 6.13 IL-12p70 production by whole blood cultures

Whole blood cultures were prepared from n = 100 umbilical cord blood (orange) and n = 103 peripheral blood of 6 month infants (green), and left unstimulated, or stimulated with LPS or PGN. The mean IL-12p70 levels in cell free culture supernatants harvested after 24 hours of culture ± SEM were measured using specific ELISA in samples that (a) had and (b) hadn't been pre-treated with 10ng/ml IFN-γ for 90 minutes. The student T was used to compare LPS and PGN induced cytokine responses to the background level. P>0.05 was considered statistically significant and denoted with. * The student T was also used to determine whether there were differences between responses from birth to 6 months of age, statistically significant differences (P>0.05) are indicated on the graph above the bracket.

6.3.4 Innate immune response in relation to probiotic versus placebo exposure

LPS- and PGN- induced IL-10 (graph not shown), IL-12p70 (Figure 6.14) and TNF-α (graph not shown) at birth and 6 months of age was investigated in PROBAT study participants. For IL-12p70, pre-treatment with IFN-γ was also considered (Figure 5.17)). IL-12p70 levels were significantly higher in the probiotic than the placebo group for the unstimulated samples at birth ($p = 0.0221$) and the LPS/IFN-γ treated samples at 6 months of age ($p = 0.0225$). There were no differences associated with probiotic supplementation for IL-10 (graph not shown) and TNF-α level under any of the cell culture conditions used at either age.

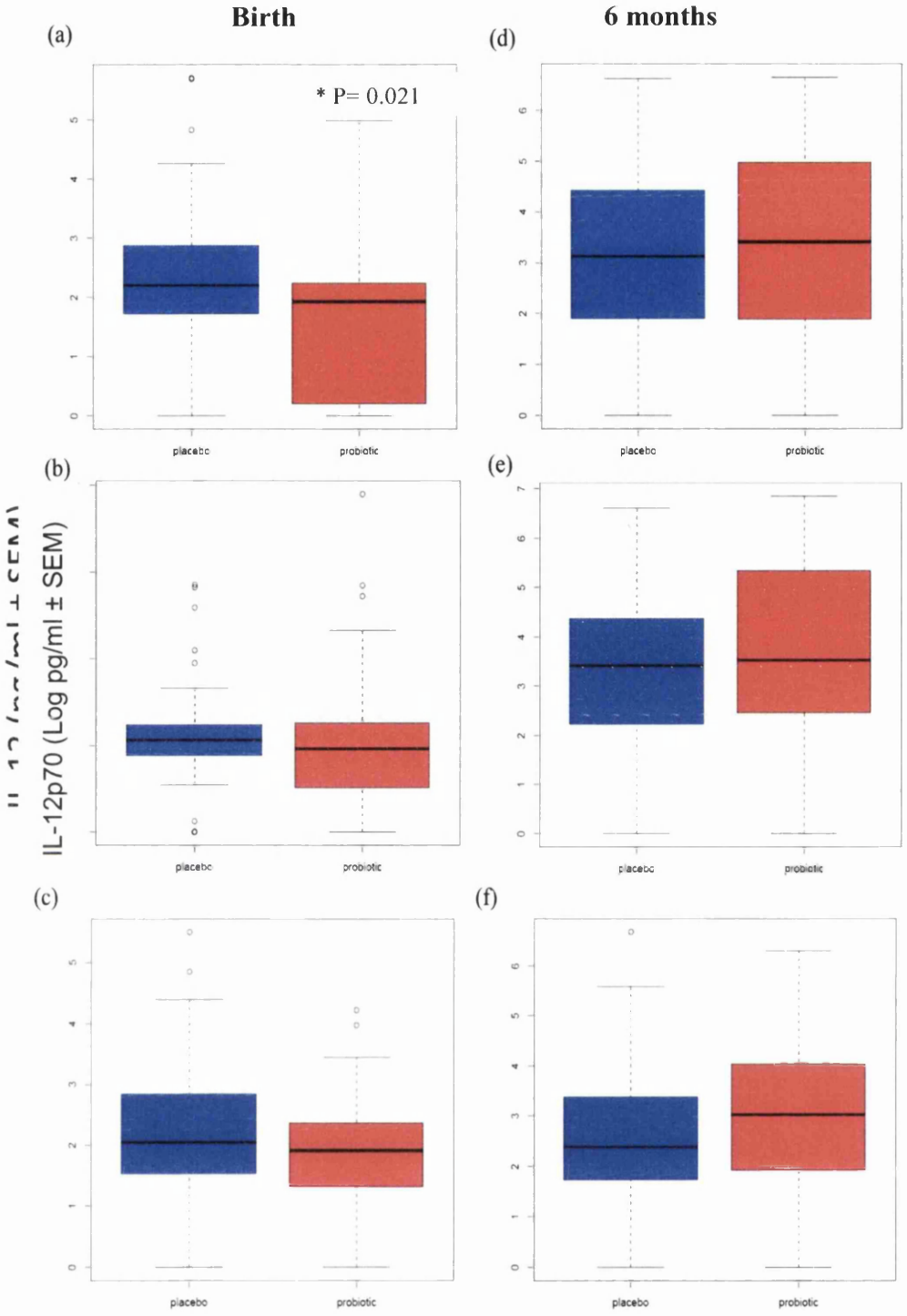


Figure 6.14 IL-12p70 response in whole blood cultures from participants in the PROBAT trial Whole blood cultures were prepared from (a,b, & c) umbilical cord blood and (d,e & f) peripheral blood of 6 month infants and (a & d) left unstimulated, or stimulated with (b & e) LPS, or (c & f) PGN. IL-12p70 levels in cell free culture supernatants harvested after 24 hour culture were measured using a specific ELISA. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. O Statistically significant results ($P < 0.05$) were denoted by *

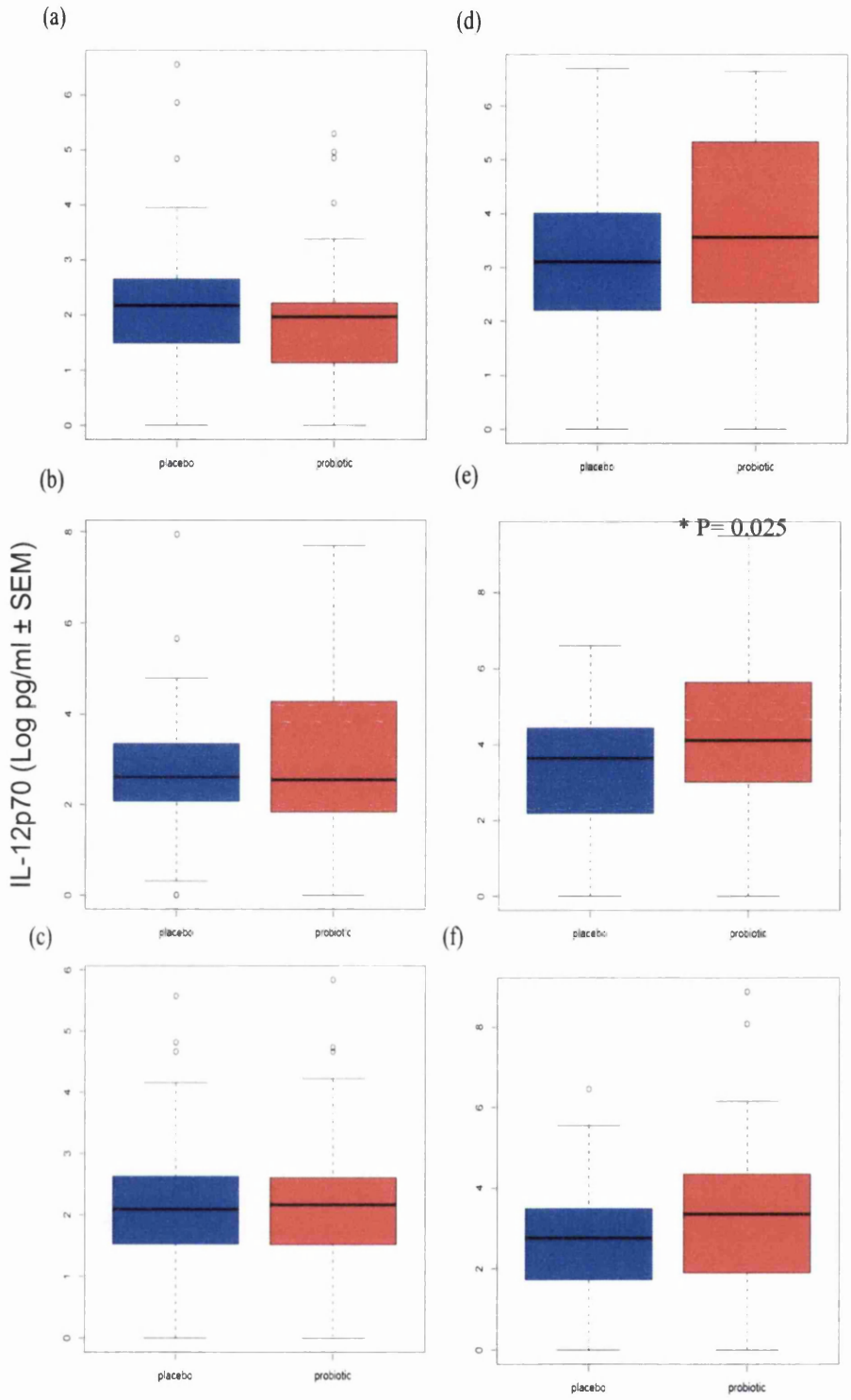


Figure 6.15 IL-12p70 response in whole blood cultures from participants in the PROBAT trial

Whole blood cultures were prepared from (a, b & c) umbilical cord blood and (d, e & f) peripheral blood of 6 month infants and pre-treated with IFN-γ (10ng/ml) for 90 minutes prior to being (a & d) left unstimulated, or stimulated with (b & e) LPS, or (c & f) PGN. IL-12p70 levels in cell free culture supernatants harvested after 24 hour culture were measured using a specific ELISA. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ○ Statistically significant results ($P < 0.05$) were denoted by *

6.3.5 Whole blood flow cytometry on umbilical cord blood and 6 month infant blood from participant of the PROBAT trial

The presentation of results takes the following format for each series of FACs analysis: explanation of the gating strategy used including an example of the key analysis plots, graphical representation of results from probiotic versus placebo analysis for birth and infant samples, and summary table of statistical analysis.

6.3.6 Lymphocyte subsets – T, B and NK cells

Lymphocytes were identified by their characteristic forward and side scatter profile (FSC and SSC, respectively) and then lineage specific antibodies – CD3 for T cells, CD19 for B cells and a combination of CD16 and CD56 for NK cells – used to identify the key lymphocyte subsets (Figure 6.18). A pan-leukocyte marker (CD45) was also included in the tube and could be incorporated into the subset identification strategy, if required. The number of each of these populations, in each μl of blood, could be calculated from data available from automated haematology analysis. There were no differences between the study groups at birth or 6 months of age for the total counts of any of these populations (Figure 6.19). However, the percentage of lymphocytes that were NK cells was significantly lower in the probiotic versus placebo group at birth ($p = 0.005$) (Figure 6.20); and the percentage that were $CD3^+$ T cells was significantly lower in the probiotic that placebo group at 6 months of age ($p < 0.0001$)

6.3.7 CD4:CD8 T cell ratio

The two major subsets of $CD3^+$ T cells are $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. Typically, the CD4/CD8 ratio is considered by firstly identifying the total $CD3^+$ subset and then the populations within this gate co-expressing either CD4 or CD8 prior to calculation of the ratio (Figure 6.21). There were no differences between the probiotic and placebo group at either time point investigated (Figure 6.22).

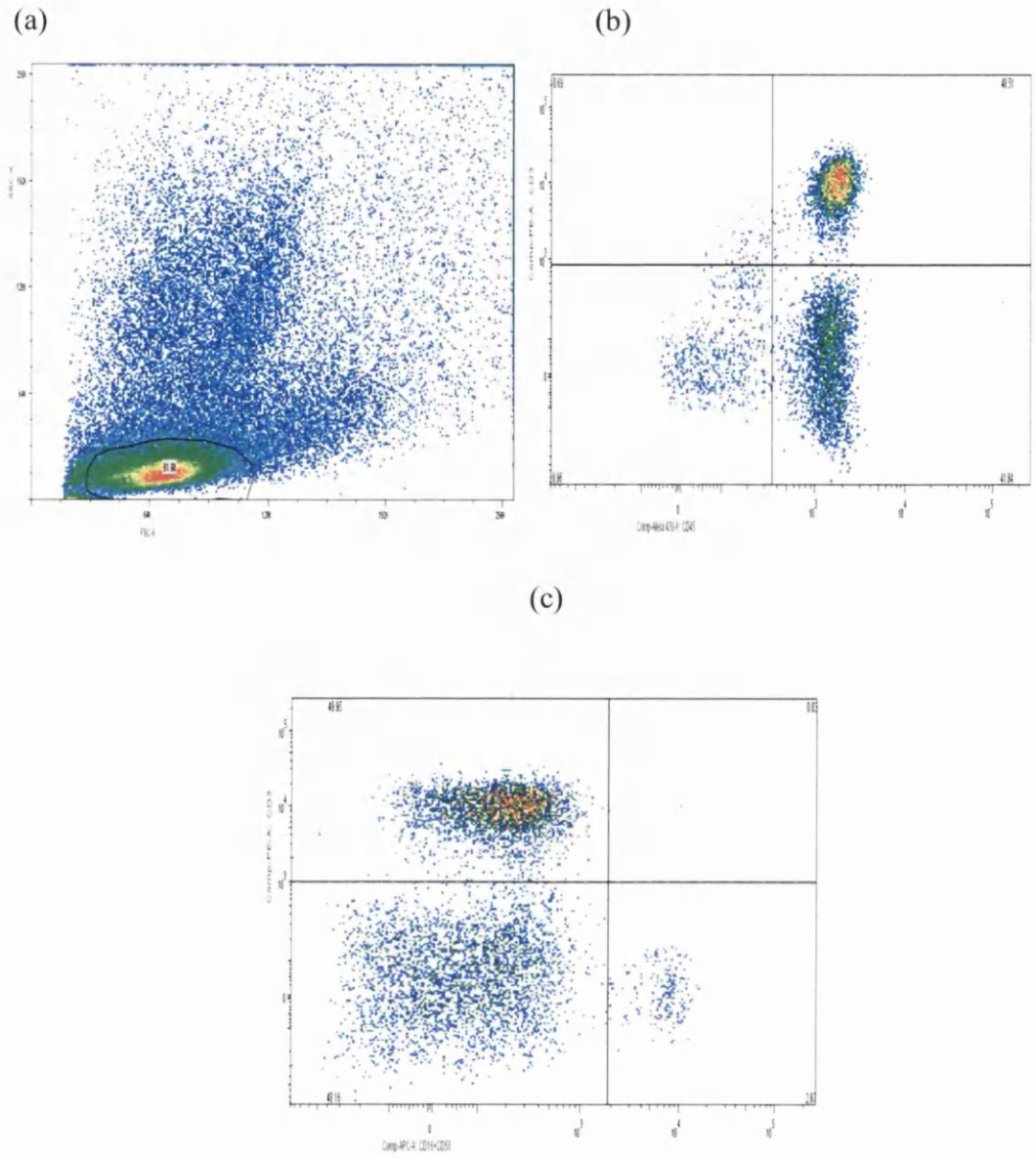


Figure 6.18: Gating strategy employed to identify key lymphocyte subsets.
From a forward scatter (FSC) versus side scatter (SSC) plot the (a) total lymphocyte population is identified by its characteristic profile. This gate is then analysed in further detail by either (b) examining CD45 expression (x-axis) versus any of the lineage markers used such as CD3 as shown (y-axis), or (c) examining expression of different lineage markers on the x-axis (in this case CD16/CD56) and the y-axis (in this case CD3). This generated data about the percentage of each key population within the total lymphocyte gate.

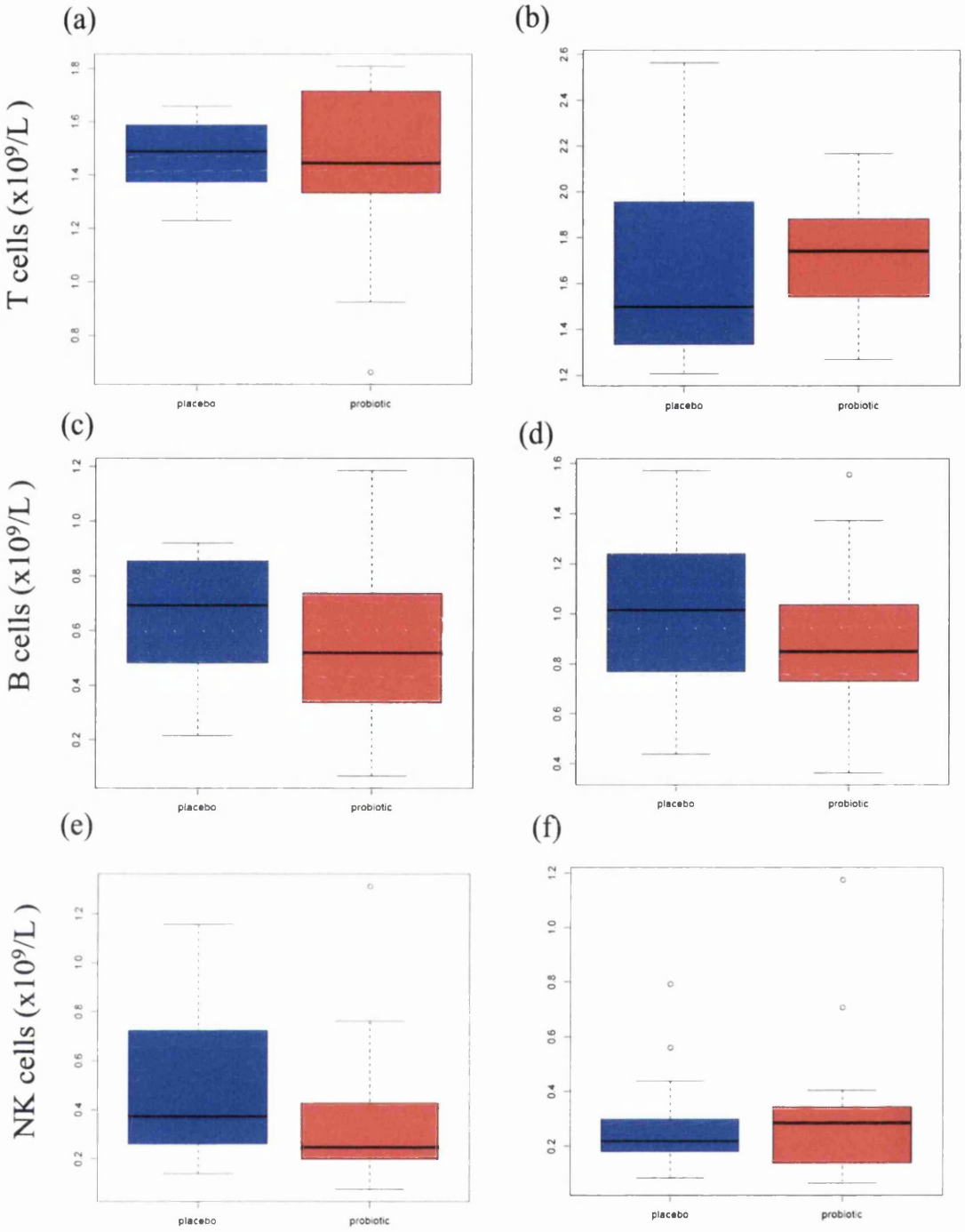


Figure 6.19: Key lymphocyte subsets by total counts at birth and 6 months of age in placebo (blue) versus probiotic (red) exposed participants of the PROBAT study.

After gating on lymphocytes the cellular count at each population ($x10^9$/L) of blood for (a & b) T cells, (c & d) B cells, and (e & f) NK cells were determined at (a, c & e) birth and at (b, d & f) 6 months of age. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

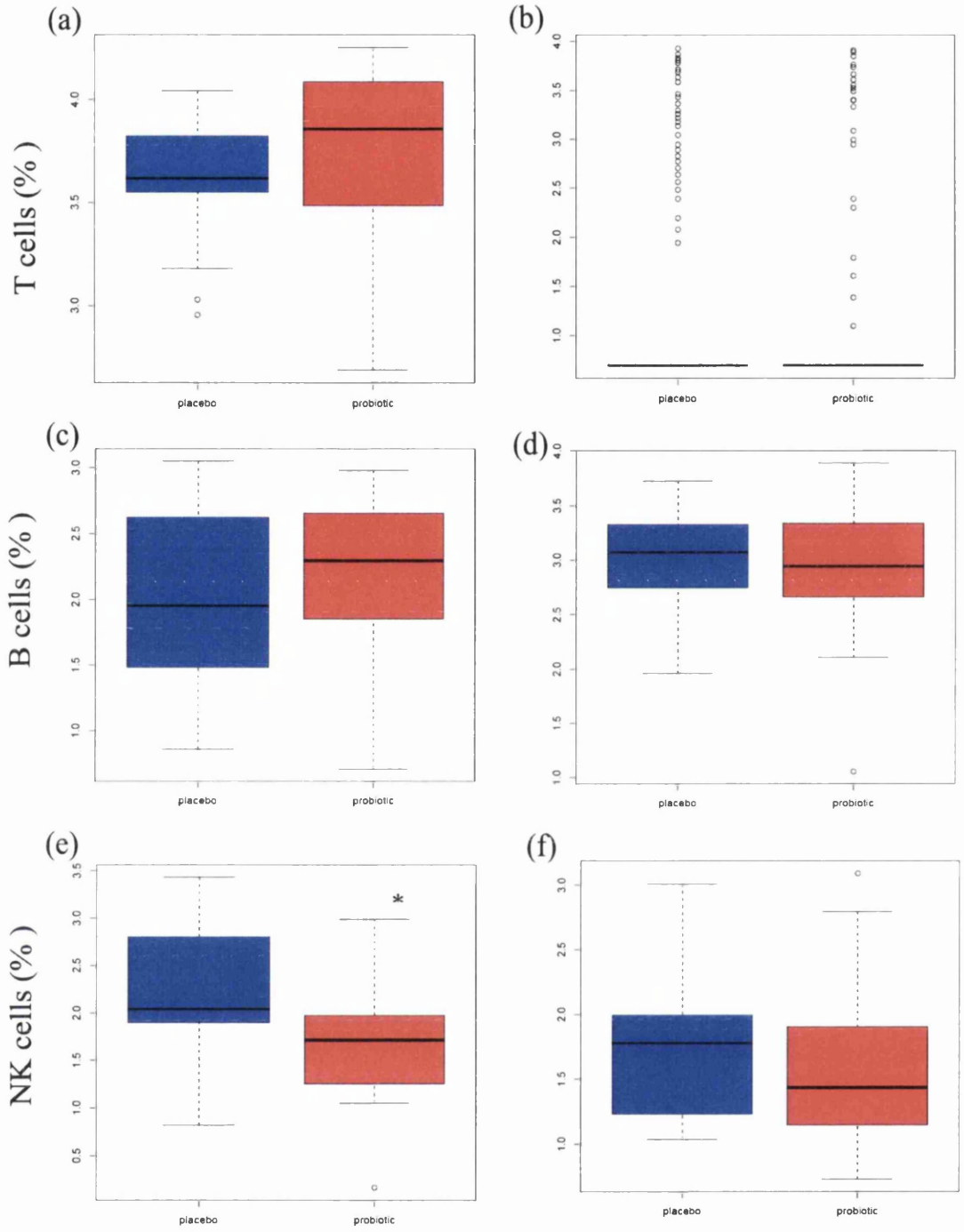


Figure 6.20: Key lymphocyte subsets by percentage at birth and 6 months of age in placebo (blue) versus probiotic (red) exposed participants of the PROBAT study.

After gating on lymphocytes the percentage of each population was determined for (a & b) T cells, (c & d) B cells, and (e & f) NK cells at (a, c & e) birth and at (b, d & f) 6 months of age. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ○ Statistically significant results ($P < 0.05$) were denoted by *

(a) (b)

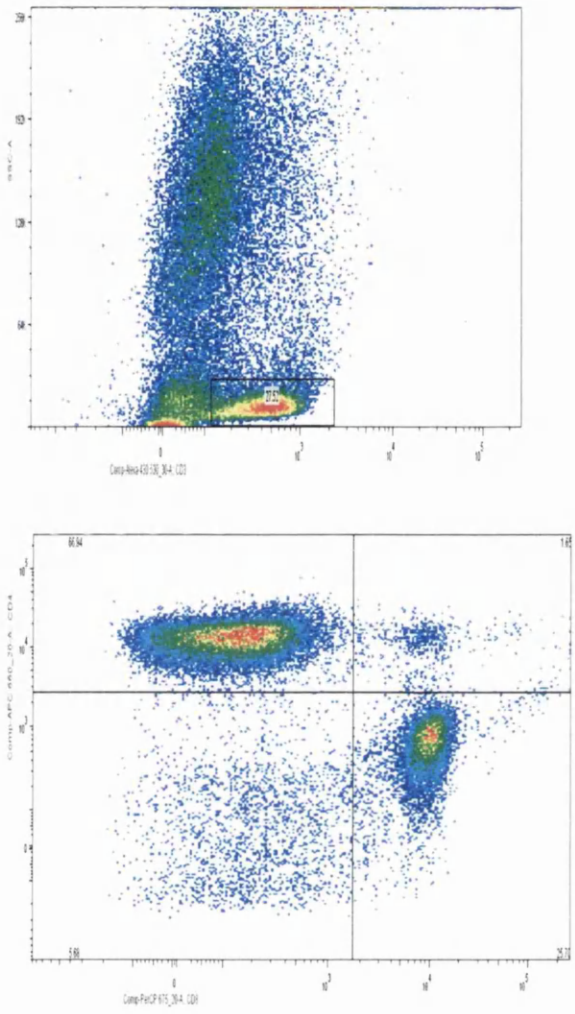


Figure 6.21: Gating strategy employed to identify $CD4^+$ and $CD8^+$ T cells.
SSC versus CD3 was used to (a) identify total $CD3^+$ T cells. This gate is then analysed in further detail by considering (b) CD8 expression (x-axis) versus CD4 expression (y-axis). This generated data about the ratio of CD4:CD8 cells within the total T cell population.

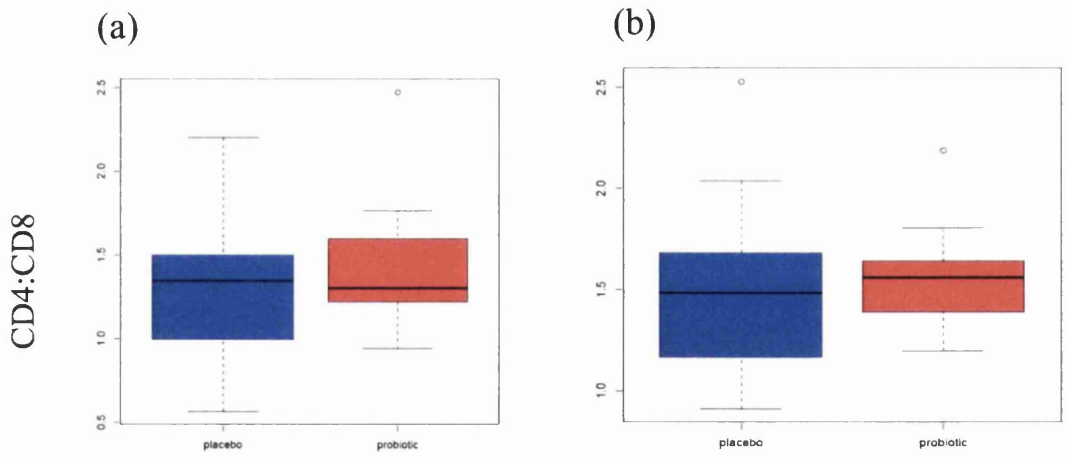


Figure 6.22: CD4:CD8 T cell ratio at birth and 6 months of age in placebo (blue) versus probiotic (red) exposed participants of the PROBAT study.
After gating on total $CD3^+$ T cells the ratio of $CD4^+$ and $CD8^+$ $CD3^+$ T cells at (a) birth, and at (b) 6 months of age was determined. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ○ Statistically significant results ($P < 0.05$) were denoted by *

6.3.8 Naïve and memory T cells

Another way of analysing T cells is to consider the relative abundance of naïve versus memory cells in which expression of CD45RA is considered to identify naïve T cells contrasting to expression of CD45RO predominantly expressed on the memory subset. In the neonate, naïve $CD45RA^+$ T cells dominate and are repeatedly reported to comprise >90% of the $CD4^+$ T cell population. Therefore after identifying $CD4^+$ cells by characteristic SSC profile and CD4 expression the percentage of cells expressing CD45RA and/or CD45RO were determined (Figure 6.24). On comparing the study groups the percentage of $CD4^+$ T cells bearing a naive phenotype ($CD45RA^+/CD45RA^-$) was significantly elevated in the probiotic group at birth ($p = 0.00739$; Figure 6.24 a). This was complemented by a significantly reduced percentage of $CD4^+$ T cells expressing a memory phenotype ($CD45RA^-/CD45RO^+$) in the probiotic group at birth ($p = 0.00875$; Figure 6.24 c). No such differences were seen at 6 months of age (Figure 6.24).

(a) (b)

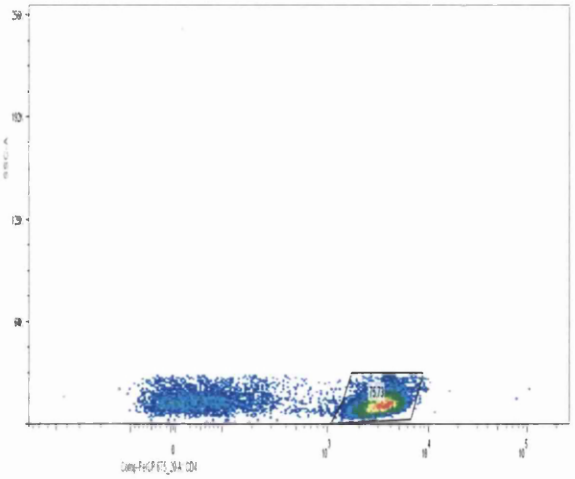
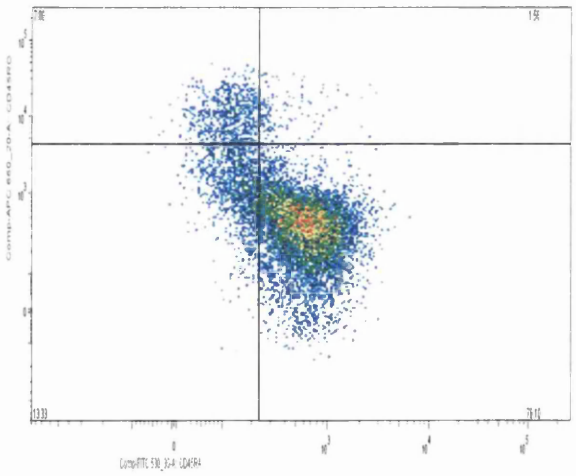


Figure 6.23: Gating strategy employed to identify CD45RA and CD45RO expressing $CD4^+$ T cells.

SSC versus CD4 was used to (a) identify total $CD4^+$ T cells. This gate is then analysed in further detail by considering (b) CD45RA expression (x-axis) versus CD45RO expression (y-axis).



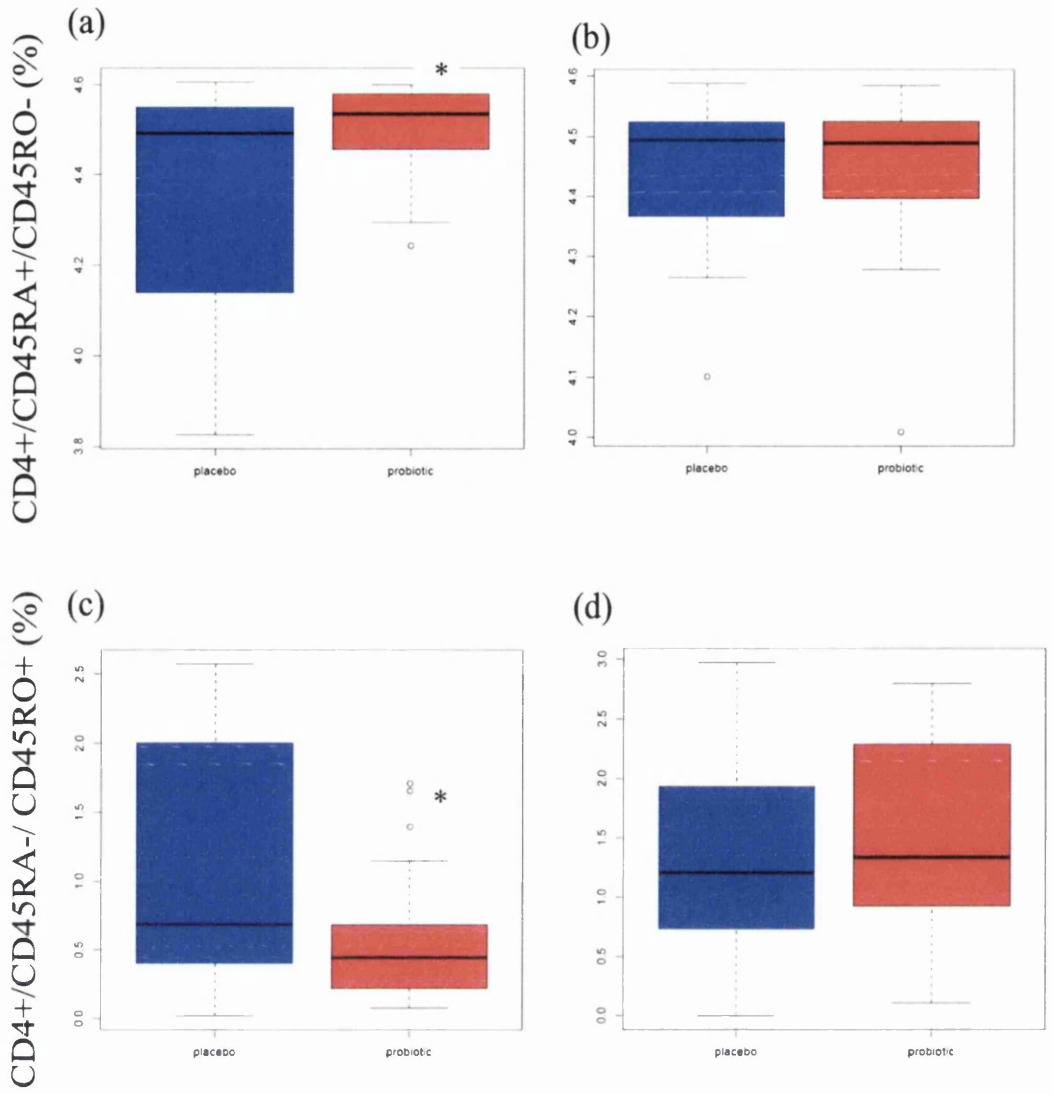


Figure 6.24: Naïve and memory CD4$^+$ T cells at birth and 6 months of age in placebo (blue) versus probiotic (red) exposed participants of the PROBAT study.

After gating on CD4$^+$ T cells the percentage of (a & b) CD45RA$^+$/CD45RO$^-$ putative naive and (c & d) CD45RA$^-$/CD45RO$^+$putative memory cells were determined at (a & c) birth and at (b & d) 6 months of age. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

6.3.8 Regulatory T cells

Putative regulatory T cells were identified as CD4$^+$ cells co-expressing CD25 (Figure 6.25). The probiotic group at birth had significantly fewer CD4$^+$CD25$^+$ T cells than the placebo group ($p = 0.00832$; Figure 6,26).

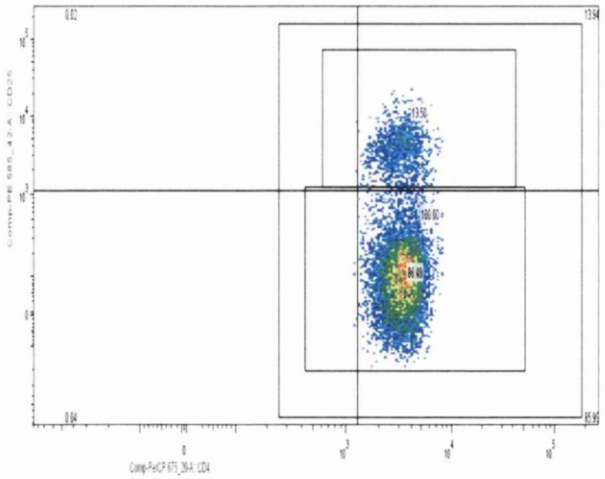


Figure 6.25: Gating strategy employed to identify $CD25^+$ $CD4^+$ T cells.

SSC versus CD4 was used to identify total $CD4^+$ T cells. This gate is then analysed in further detail by considering CD25 expression (y-axis).

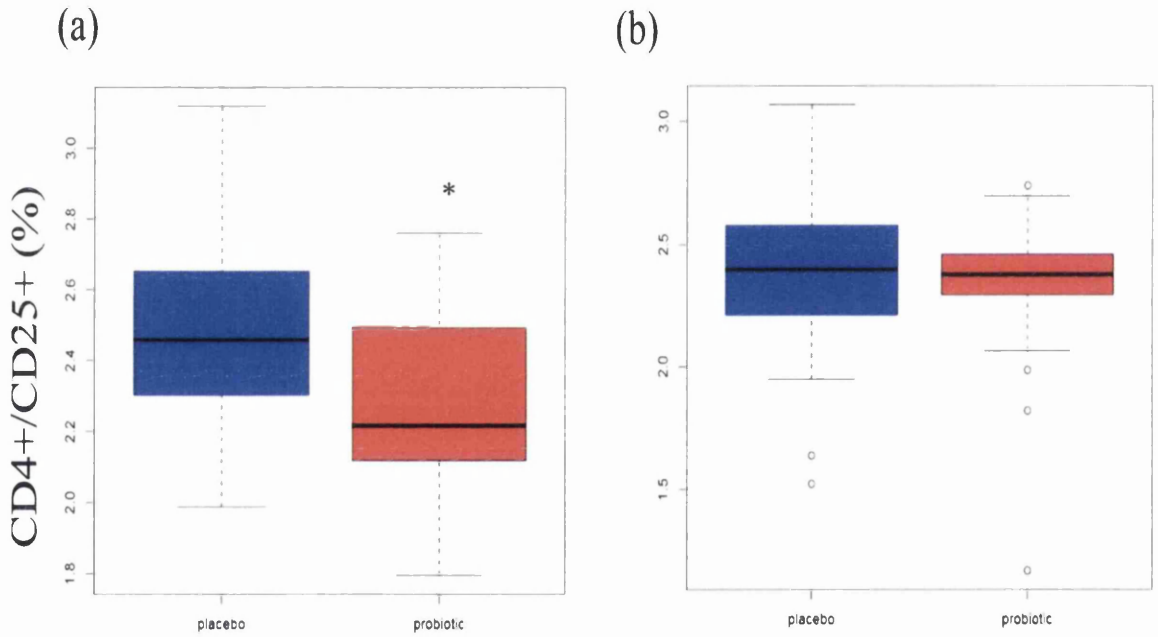


Figure 6.26: CD25 expressing $CD4^+$ T cells at birth and 6 months of age in placebo (blue) versus probiotic (red) exposed participants of the PROBAT study.

After gating on $CD4^+$ T cells the percentage of $CD25^+$ cells at (a) birth, and at (b) 6 months of age was determined. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

5.3.9 Gut homing T cells

Since probiotic immunomodulatory effects are propagated in the gut, the analysis of putative gut homing T cells in the circulation was considered as a surrogate of immunological activity in the gut. Expression of the integrins α4 (CD49d) and β7 (CD29) on both $CD4^+$ and $CD8^+$ T cells was examined using flow cytometry (Figure 6.27). Perinatal probiotic supplementation was associated with reduction of $CD8^+$ T cells expressing at birth (β7 alone – $p = 0.00898$, Figure 6.29 a) and both β7/CD49d – $p = 0.00501$, Figure 6.28 c).

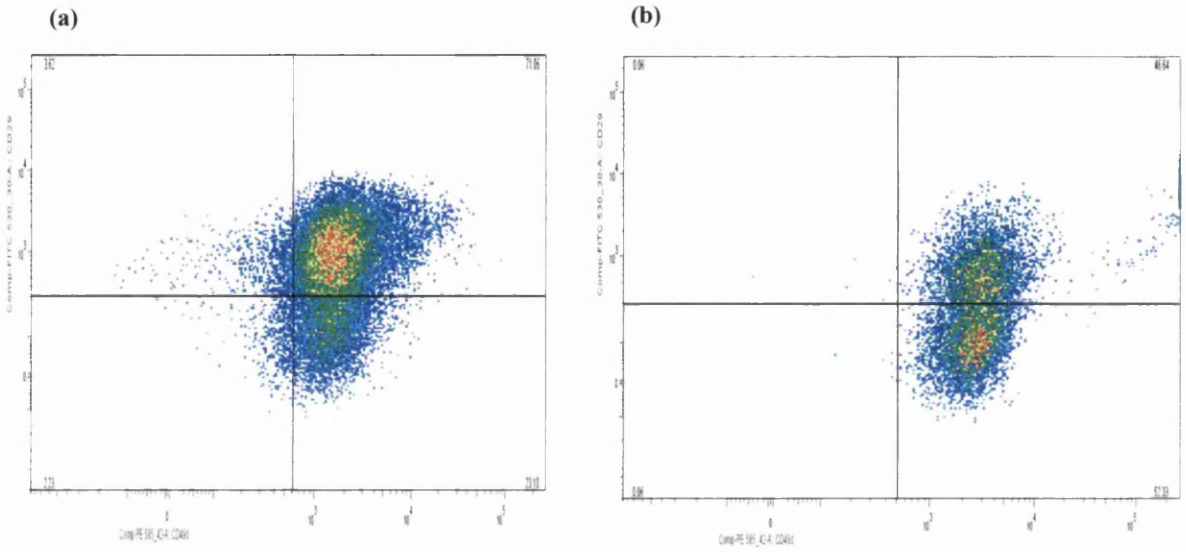


Figure 6.27: Gating strategy used to identify putative gut homing $CD4^+$ and $CD8^+$ T cells.
$CD4^+$ and $CD8^+$ T cells were first identified and expression of CD49d (x-axis) and β7 integrin (CD29; y-axis) was determined for (a) $CD4^+$ and (b) $CD8^+$ T cells.

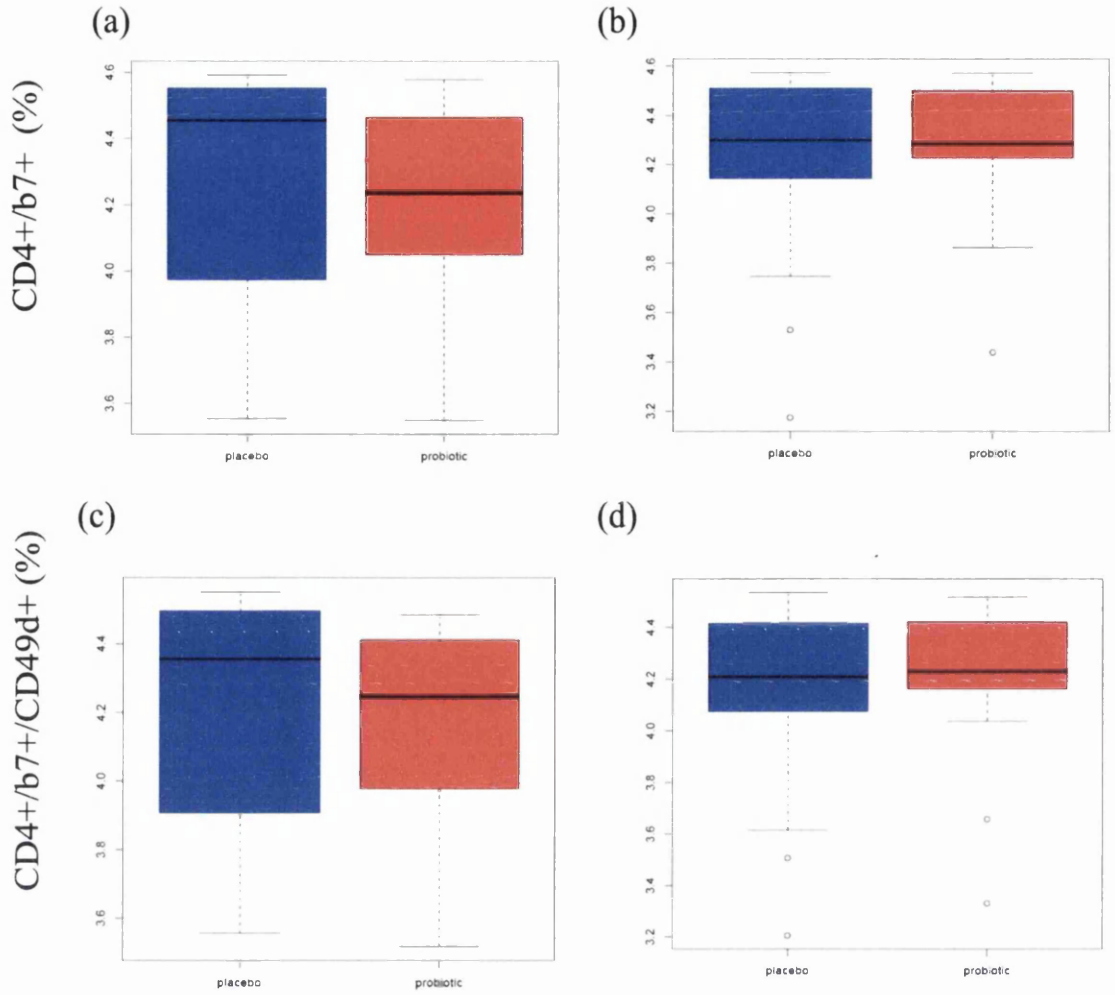


Figure 6.28: β7 and CD49d expressing $CD4^+$ T cells at birth and 6 months of age in placebo (blue) versus probiotic (red) exposed participants of the PROBAT study.

After gating on $CD4^+$ or T cells the percentage of (a & b) β7 expressing, and (c & d) β7/CD4d co-expressing cells were determined at (a & c) birth and at (b & d) 6 months of age. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ○ Statistically significant results ($P < 0.05$) were denoted by *

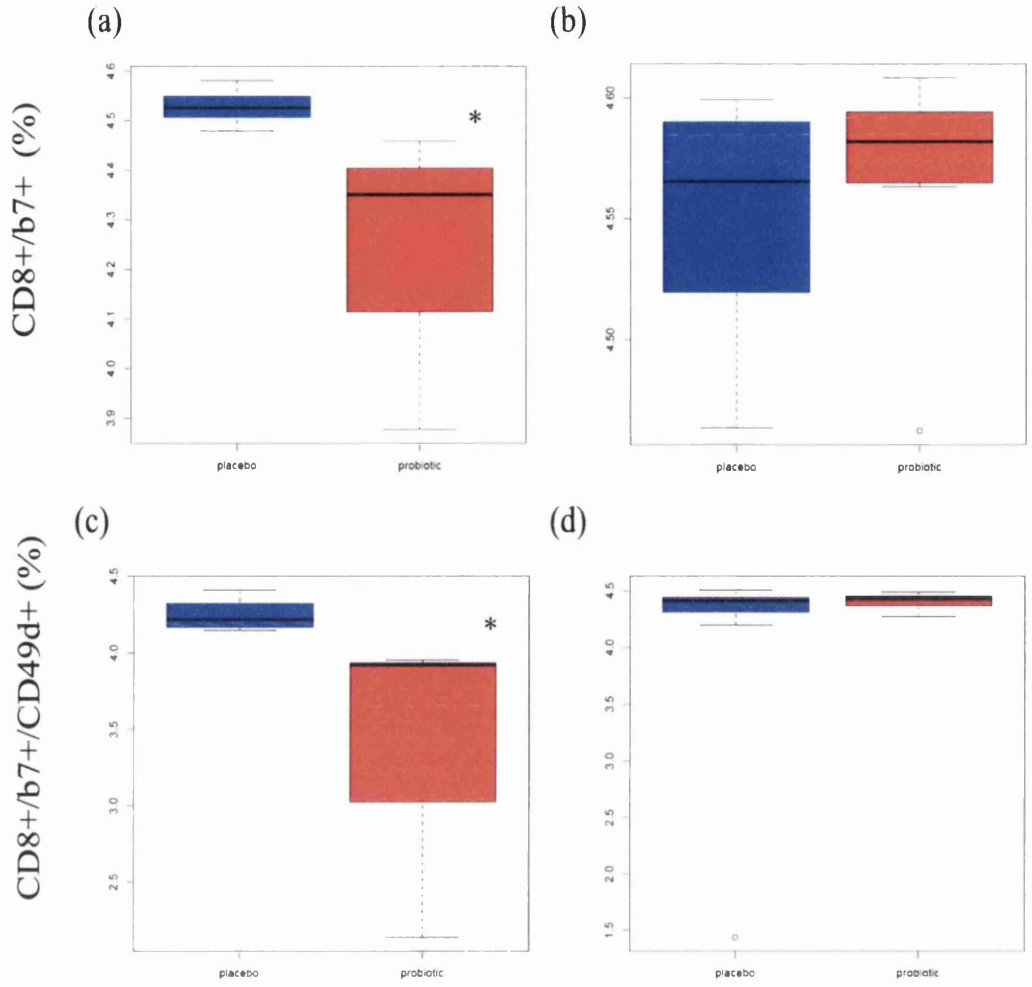


Figure 6.29: β7 and CD49d expressing $CD8^+$ T cells at birth and 6 months of age in placebo (blue) versus probiotic (red) exposed participants of the PROBAT study.

After gating on $CD8^+$ or T cells the percentage of (a & b) β7 expressing, and (c & d) β7/CD49d co-expressing cells were determined at (a & c) birth and at (b & d) 6 months of age. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

6.3.10 B cells

B cells were identified by their typical SSC and expression of the B lineage marker CD19 expression of other CD antigens of interest – CD21, CD23, IgM, CD1c and CD5 – was determined by gating on this population and determining the percentage positive cells using a fluorochrome-conjugated antibody to the surface antigen of interest versus an isotype control (Figure 5.30). The percentage of $CD19^+$ B cells expressing the surface markers of interest did not differ between the two study groups at either birth or 6 months of age (Figure 6.31 and 6.32).

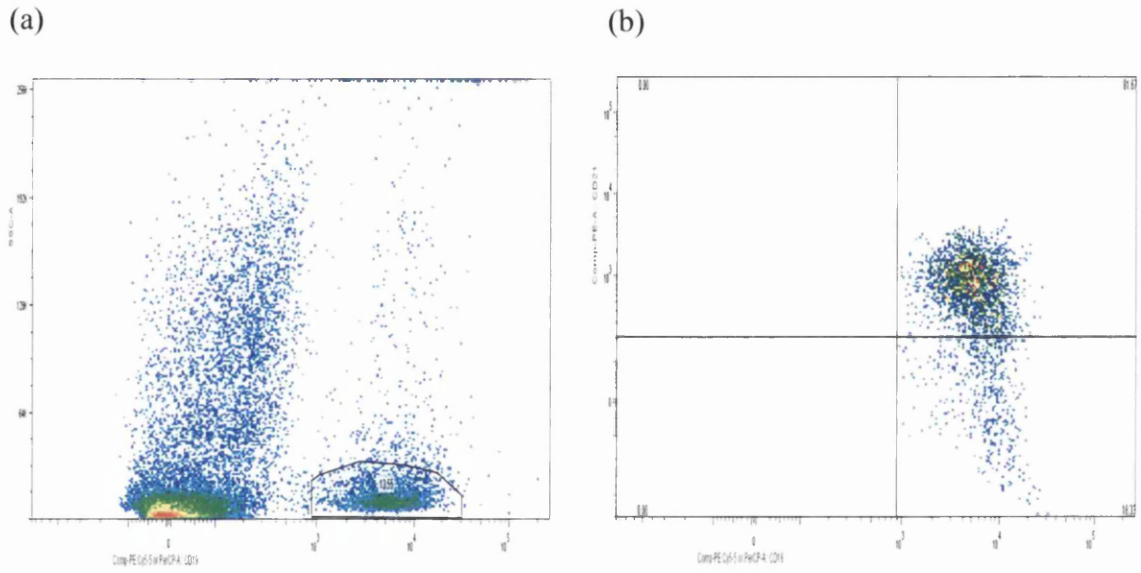


Figure: 6.30 Gating strategies employed to identify $CD19^+$ B cells expressing CD21, CD23, IgM, CD1c or CD15.
Total B cells were first identified by (a) SSC versus CD19. After gating on these cells further analysis for expression of other markers of interest such as (b) CD21 (y-axis) was determined.

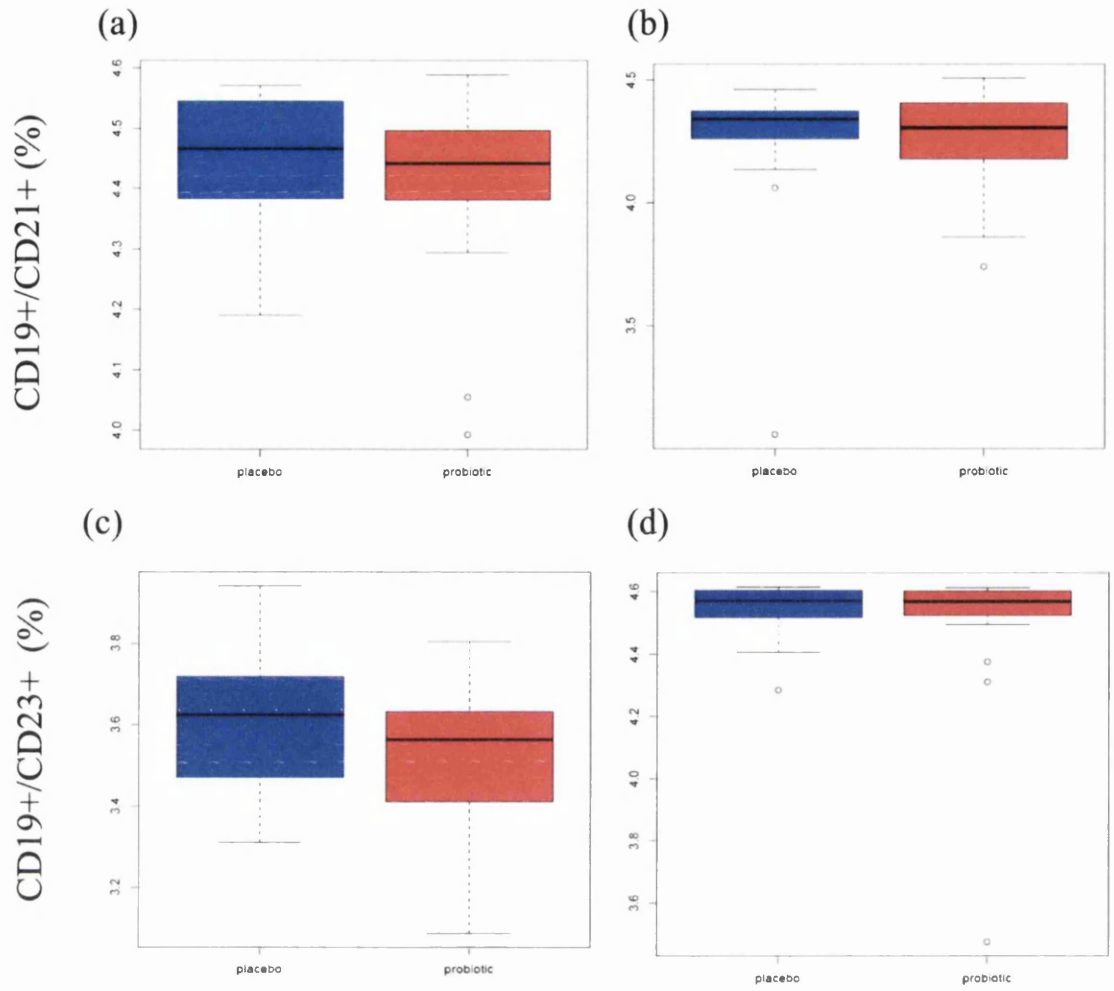


Figure 6.31: CD19$^+$ B cells at birth and 6 months of age in placebo (blue) versus probiotic (red) exposed participants of the PROBAT study.
After gating on CD19$^+$ B cells the percentage of cells expressing (a & b) CD21, and (c & d) CD23 was determined at (a & c) birth and at (b & d) 6 months of age. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

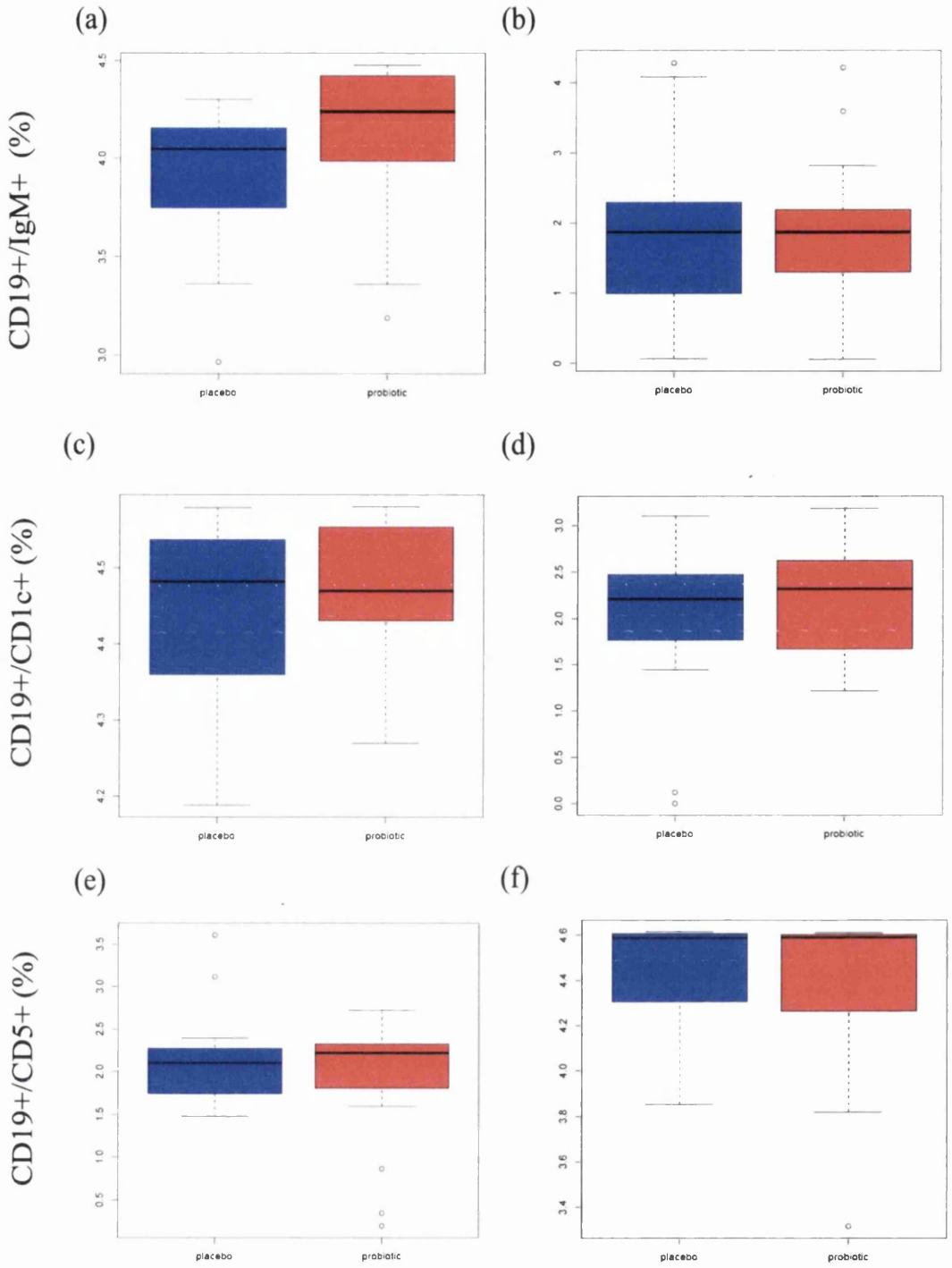


Figure 6.32: CD19$^+$ B cells at birth and 6 months of age in placebo (blue) versus probiotic (red) exposed participants of the PROBAT study.
After gating on CD19$^+$ B cells the percentage of cells expressing (a & b) IgM, (c & d) CD1c, and (e & f) CD5 was determined at (a, c & e) birth and at (b, d & f) 6 months of age. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

6.3.11 Dendritic cells

Dendritic cells were identified by their lack of range of lineage markers (including CD3, CD14 and CD19) but expression of HLA-DR (Figure 6.33a). This also allowed calculation of the percentage of leukocytes that were dendritic cells (total DCs). The two major subsets of DCs could then be identified using antibodies to CD123 (IL-3Ra) and CD11c. This enabled identification of plasmacytoid DCs (pDCs; $CD123^{+}/CD11c^{-}$) and myeloid DCs (mDCs; $CD123^{+}/CD11c^{+}$) (Figure 6.33b) and calculation of each of these populations as a percentage total DCs. There were no differences in total DCs or either of the subsets at birth or 6 months in those taking probiotics (Figure 6.34).

(a) (b)

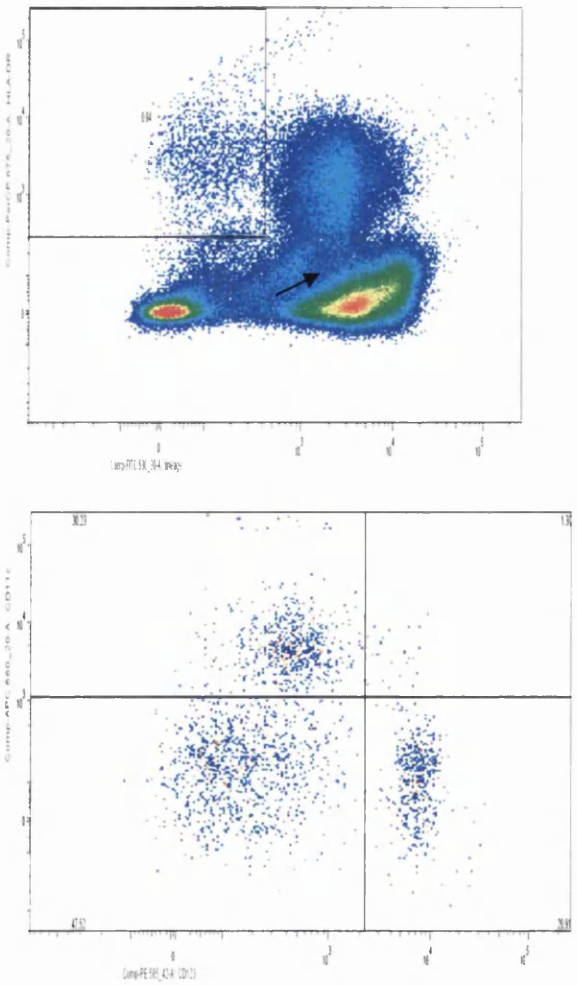


Figure 6.33: Gating strategy for identification of dendritic cells subsets.

Total dendritic cells were identified as (a) lineage-negative (x-axis) and HLA-DR-positive (y-axis). Further analysis of this gated population was undertaken with (b) anti-CD123/IL-3R (x-axis) and CD11c (y-axis) to identify plasmacytoid dendritic cells ($CD123^{+}/CD11c^{-}$) and myeloid dendritic cells ($CD123^{-}/CD11c^{+}$).

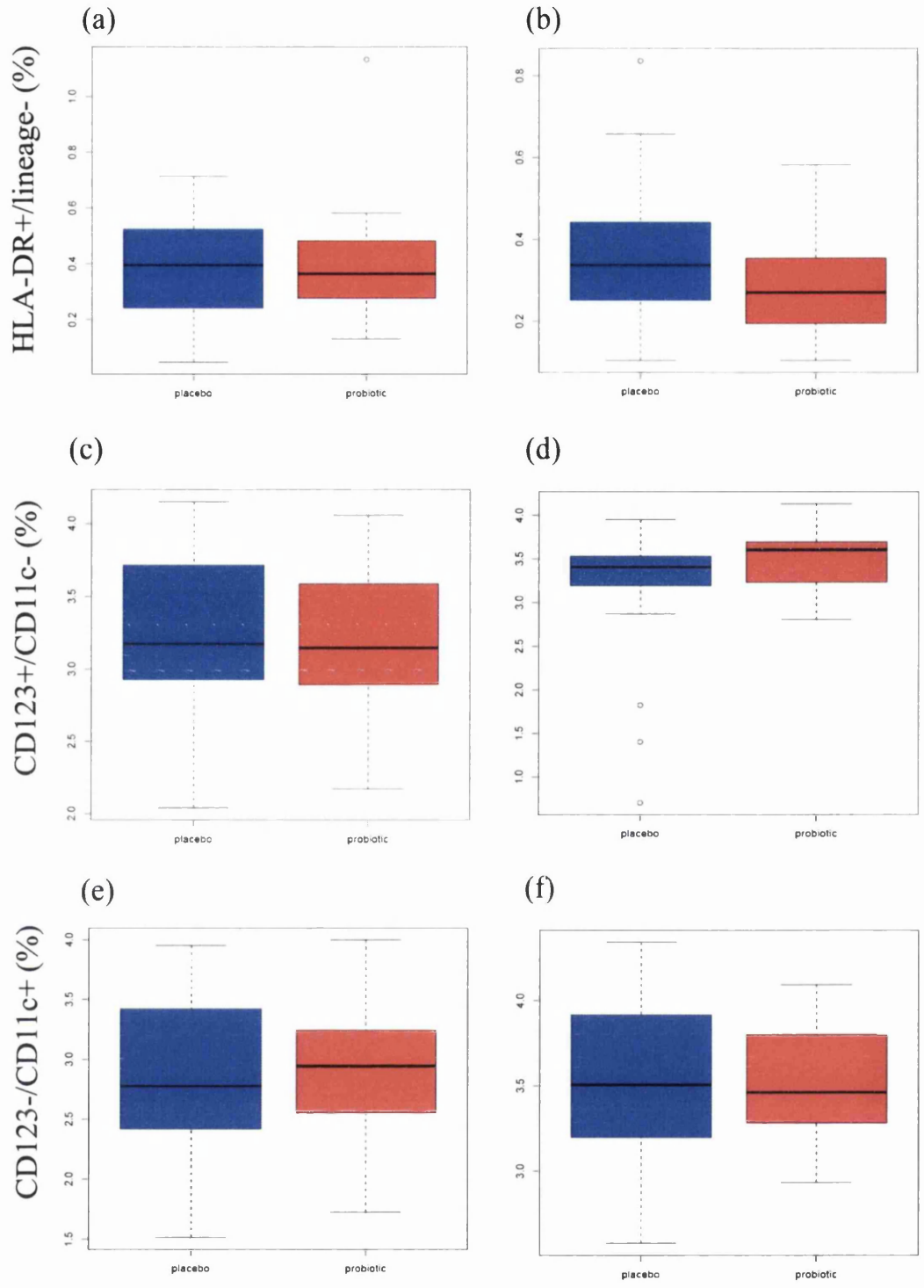


Figure 6.34: Total and subsets of dendritic cells at birth and 6 months of age in placebo (blue) versus probiotic (red) exposed participants of the PROBAT study.

Flow cytometry was used to identify (a & b) total DCs, (c & d) pDCs, and (e & f) mDCs at (a, c & e) birth and at (b, d & f) 6 months of age. The box signifies the upper and lower quartiles (75% and 25% respectively), and the median is represented by the thick black line within the box. The whiskers represent the 5% and 95% percentile. Outliers are represented by. ^{O} Statistically significant results ($P < 0.05$) were denoted by *

6.4. Discussion

6.4.1 Age dependent cellular maturation of neonatal immunity from birth to 6 months of age

The *in vivo* immunomodulatory influence of perinatal probiotic supplementation during development of the newborn immune system from birth to 6 months of age was assessed during two fundamental aspects of neonatal immunity: (1) Cellular ontogeny of newborn haematopoiesis at the cell population level and (2) Augmentation of neonatal innate immune function towards microbial stimuli. In this discussion, special reference is given to how the observed effects of supplementation might relate to the onset of IgE mediated atopic disorder in the neonate.

Results of the current study highlighted statistically significant cellular differences between birth and 6 months of age in cord blood for total white blood cells, neutrophils, monocytes, eosinophils, basophils and platelets. With these cell types consistently more abundant in cord blood samples compared to infant blood samples, suggesting that granulopoesis is favoured in the neonate with a shift towards lymphopoiesis during infancy. These observations were supported by the fact that lymphocytes were significantly higher only in the 6 month infant samples. Matching of the birth and 6 month infant blood samples confirmed these dramatic differences in lymphocyte and granulocyte populations among the subsets studied (Figure 6.2; 6.3). These observations were in line with observations by Pranke *et al.* (2001), Christensen *et al.* (2009) and Erkeller-Yuksel *et al.* (1992) who measured changes in major lymphocyte populations as a function of age and demonstrated that percentage lymphocyte counts increased from 41% at birth in cord blood to 47% between 2 days to 11months, only decrease by 1% during the period of 1 yr. to 6 yrs. and by 7% between 7 and 17 years.

From a qualitative standpoint, using the neutrophil as a characteristic example, although newborns have a reduced number of quiescent granulocyte and monocyte progenitor cells and a diminished precursor storage pool, neonatal neutrophils show impairment of multiple functional aspects, including chemotaxis, transmigration and rolling adhesion (Carr 2000; Henneke and Berner 2006). Such migratory defects correlate with the high proportion of immature neutrophils we have observed in the

present study in umbilical cord blood. Furthermore functional deficits of neonatal neutrophils, such as diminished L-selectin expression and leukocyte migration into tissues, have been observed for neonatal eosinophils such that eosinophils are not normally resident in bodily tissues (Smith *et al.* 1992). Interestingly cord blood monocytes also exhibit chemotaxis defects (Holt *et al.* 2005; Tulic *et al.* 2011).

6.4.2 Cell counts in relation to probiotic versus placebo exposure

To date since 1997 over 30 randomised double-blind placebo-controlled clinical trials studying the efficacy of various probiotic combinations to prevent the induction of IgE-mediated allergy have been conducted. This is one of the first perinatal supplementation studies to assess the effects of a consortium of probiotic bacteria on the cellular context of granulopoesis. Interestingly, Allen *et al.* (2014) discovered a statistically significant reduction in eosinophil counts at birth in infants whose mothers had taken the probiotic supplement over the last few weeks of pregnancy. Since eosinophils mature only in the presence of the IL-5, IL-3 and GM-CSF this current observation is promising when reviewed in the light of clinical data that elevated eosinophil counts at birth and/or at 3 months are associated with early diagnosis of eczema and positive skin prick tests at 18 months of age (Borres *et al.* 1995; Borres and Bjorksten 2004; Matsumoto *et al.* 2005). The current study suggests that perinatal probiotic supplementation might provide a Th1 driven immunomodulatory signal capable of curtailing the differentiation of eosinophil. We hypothesised that this effect might require signals transmitted via gestation-associated tissues, such as the placenta (Conrad *et al.* 2009; Thornton *et al.* 2010).

These results were indirectly consistent with a probiotic treatment study conducted by Rosenfeldt *et al.* (2003), who administered (lyophilized) *Lactobacilli rhamnosus* 19070-2 and *Lactobacilli reuter*i DSM 122460) given in combination for 6 weeks to 1- to 13-year-old children afflicted with Atopic dermatitis, and employed the use of eosinophil cationic protein (ECP), a cytotoxic protein released from activated eosinophils, as a proxy to monitor disease activity in AD. During active treatment, serum (ECP) levels significantly decreased as a result of supplementation, suggesting as in the current study that the immunomodulatory effect of probiotic bacteria impacts upon eosinophil activity. Another study conducted by Brouwer *et al.* (2006) showed, during lactobacilli species supplementation, a moderate but significant reduction in soluble ECP levels was found.

6.4.3 Innate immune response at birth and 6 months of age

The current study served to determine whether the immunological immaturity of newborn immunity marked by an innate immune bias towards upregulation of potentially atopic Th2 polarising cytokine responses (de Vries *et al.* 2000) can be reversed via perinatal probiotic supplementation. The principle observations were that LPS and PGN induced statistically significant age-dependent maturation of IL-10 and TNF-α response with age by whole blood cultures with these responses greater at 6 months of age. This age dependent maturation was consistent with results presented in the literature indicating that IL-10 induction during the neonatal period increases with age. Additionally, polarisation of TLR-Mediated IL-10 responses of neonatal cord blood derived monocytes and APCs was increased during the neonatal period, with IL-10 production in the neonate three- to fivefold more than adults (De Wit *et al.* 2003; Chelvarajan *et al.* 2004).

Interestingly, the current study reported a statistically significant increase in TNF-α at 6 months of age in response to LPS and PGN at a time when induction of a TNF-α response is attenuated and tightly regulated.

Consistent with the age dependent increases in Th1 pro-inflammatory cytokines between birth and 6 months of age, IL-12p70 levels in unstimulated, PGN and IFN-γ stimulated whole blood cultures were significantly elevated at 6 months of age compared to birth, indicating an age dependent maturation of IL-12p70. This data was consistent with studies by Prescott *et al.* (2011) who sought to explore whether allergic children show differences in microbial TLR-mediated responses over their first 5 years of life. Interestingly they observed in non-allergic children a progressive and significant age-related increase in innate cytokine responses (IL-1β, IL-6, TNF-α, and IL-10) to virtually all TLR ligands corresponding with a parallel increase in adaptive Th1 (IFN-γ) responses to allergens and mitogens. Contrastingly, allergic children show exaggerated innate responses at birth but a relative decrease with age thereafter, so that by age 5 years, TLR responses are significantly attenuated compared to non-allergic subjects. It was concluded that early hyper-responsiveness in allergic subjects failed to translate to an equivalent maturation of Th1 function,

which remained attenuated relative to non-allergic subjects but associated with an aberrant age-dependent statistically significant increase in allergen-specific Th2 responses.

6.4.4 Innate immune response in relation to probiotic versus placebo exposure

Probiotic bacteria augment maturational signals for development of the neonatal gut-associated lymphoid tissue *in vivo* (Hooper and Macpherson 2010). Additionally, their immunogenicity upon interaction with the cellular components of neonatal immunity renders them key contributors to the pro- and anti-inflammatory cytokine immune equilibrium within the neonatal gut. To test this hypothesis, we stimulated whole blood cultures at birth and 6 months of age from recruits on the PROBAT trial with the microbial stimuli LPS and PGN, and measured the ensuing innate and adaptive cytokine responses.

Perinatal probiotic supplementation significantly increased production of the Th1 cytokine IL-12p70 in the probiotic group in unstimulated whole blood culture samples at birth. This type of response was indirectly consistent with the secondary outcomes of the trial of Allen *et al.* (2014) who reported that perinatal probiotic supplementation was synonymous with a reduction in IgE-mediated atopic sensitisation. This is because IgE-mediated atopic sensitisation cannot develop in an immunological microenvironment that is Th1 orientated. Furthermore, LPS/IFN-γ induced IL-12p70 responses by whole blood cultures were also significantly increased in the probiotic group at 6 months of age compared to the placebo group further supporting the notion that perinatal probiotic supplementation enhances maturation of the Th1 immune response *in vivo.* These results were also in keeping with the notion that probiotic induction of a low-grade inflammatory response might contribute to their efficacy in preventing atopy (Marschan *et al.* 2008). To further explore this in the PROBAT trial cohort, in the future we aim to analyse faecal samples and plasma/serum samples to compare local gut and systemic inflammatory status as a consequence of perinatal probiotic supplementation.

The current results were in line with the results of West *et al.* (2009) from an immunological standpoint despite differences in their experimental approach. In a double-blind, placebo-controlled randomized intervention trial infants were fed

cereals with (n: 89) or without *Lactobacilli* F19 (n: 90) from 4 to 13 months of age and the incidence of eczema and Th1/Th2 balance evaluated during weaning. At 13 months of age, the authors demonstrated that IFN-γ/ IL-4 mRNA ratio was significantly higher in the probiotic compared with the placebo group. The higher Th1/Th2 ratio in the probiotic compared to placebo group suggests enhancing effects of *Lactobacilli* F19 on the T cell-mediated immune response. Probiotics also increased Th1 cytokines and inhibited allergen-induced IgE and Th2 cytokines in some atopic children. In another study Pohjavuori *et al.* (2004), showed an increase in IFN-γ production in peripheral blood mononuclear cell in infants with Atopic dermatitis (AD) treated with the probiotic LGG instead of placebo concomitant with an improvement in AD severity associated with significant increases in the capacity for Th1 IFN-γ responses. This effect was still evident 2 months after the supplementation was ceased.

The results of the present study, support the ability of the study consortium to augment Th1 responses *in vivo* and perhaps accelerate maturation of an otherwise attenuated Th1 pro-inflammatory cytokine pathway during the neonatal period. Whether or not the observed Th1 immune responses can be linked to protection against IgE-mediated atopic disorder will be reviewed in the light of the clinical outcomes of the PROBAT trial. Promisingly Th1 polarising cytokine responses are antagonistic to the up-regulation of IgE mediated Th2 atopic responses *in vivo* and contribute to the polarisation of naive T cell towards the Th1 phenotype.

In contrast to the results of the current study, in a randomized prenatal controlled trial by Boyle *et al.* LGG, treatment during pregnancy for the prevention of eczema was not associated with any change in the cord blood immune markers IL-10, IL-12, IFN-γ and TNF-α selected. There observations may be in part due to the mode of probiotic supplemental scheme, suggesting that there may be differences in observed immunological outcomes between perinatal versus prenatal probiotic supplemental schemes

Data from the current study provided further support for the application of probiotic bacteria as a strategy to attenuate the risk of IgE-mediated atopic disorder (Kaliomaki *et al.* 2001, 2007; Niers *et al.* 2009) at both the cellular and innate immune level during early infancy. The application of haematological analyses to

monitor alterations in both granulocytic and lymphocyte subsets from genetically predisposed atopic individuals combined with research into the underlying immune function of these subsets provided an in valuable diagnostic and prognostic research tool for further understanding the pathogenesis of atopic disorder; and the relationship between the microbial influence and the likelihood of immune dysregulated disorders in later life (Raes *et al.* 1993).

Despite the usefulness of data evaluating the cellular ontogeny of granulopoeisis and lymphopoiesis in the neonate compared to 6 month old infant, information on factors that affect these processes are limited. Since optimisation of the newborn intestinal microbiota via perinatal probiotic bacteria supplementation with intestinal bacteria of lactobacilli and bifidobacterial origin has been cited numerously as a method to both galvanise the integrity of newborn immunity and as a strategy to attenuate the risk of IgE-mediated atopic disorder; it seemed logical to determine whether probiotic bacteria forming an important constituent of the newborn microbiota might enhance process of granulopoeisis and lymphopoiesis in early life. The results of the current study indicate that the consortium plays an immunomodulatory role during age-dependent maturation of innate and adaptive cytokine responses from birth to 6 months of age. However perinatal probiotic supplementation only had a transient effect on granulopoesis, effecting only the eosinophil population involved in the pathogenesis of atopic disorder at the blood circulation level at birth. Further analysis of the PROBAT study outcomes is required to determine whether this observation can be linked to a reduction in atopic disorders such as eczema, where eosinophilia at the sites of allergic inflammation is a hallmark component. Germ free animal models indicate that lymphocyte subset maturation during the neonatal period is influenced by the immunogenicity and/or types of probiotic organisms colonising the intestinal tract, upon interaction with the GALT. The key findings are now discussed:

6.4.5 Effects of perinatal probiotic supplementation on naïve and memory T lymphocyte subsets

Flow cytometric analysis indicates that T cell compartments undergo dramatic compositional changes during childhood and that naïve T cells are not generated exclusively by the thymus but also via cytokine-mediated proliferation of naïve T cells within the periphery (van Gent *et al.* 2009). We considered the immunomodulatory role of perinatal probiotic supplementation in this process and

discovered that the percentage of $CD3^+$ T cells (employed as a surrogate marker for T cell activity) was significantly lower in the probiotic than placebo group at 6 months of age ($p < 0.0001$). This observation might imply that the immunomodulatory effect of perinatal probiotic supplementation renders the neonate less prone to antigenic challenge posed by allergic or infectious threats, therefore obviating the need for a robust adaptive immune response to less harmful pathogenic or allergic challenge.

Although information regarding the CD4/CD8 ratio is not typically studied in atopic disorder, Joffe *et al.* (1983) demonstrated that the CD4/CD8 ratio increases in atopic dermatitis due to an increase of $CD4^+$ T cells. Perinatal probiotic supplementation did not seem to have an effect on the CD4/CD8 ratio at either time point investigated in the current study.

At birth there is a natural predominance of $CD45RA^+/CD45RO^-$ 'naive' T cells as the principal neonatal population (van Gent *et al.* 2009). On comparing the study groups, the percentage of $CD4^+$ T cells bearing a naive phenotype ($CD45RA^+/CD45RO^-$) was significantly elevated in the probiotic group at birth. This was complemented by a significantly reduced percentage of $CD4^+$ T cells expressing a memory phenotype ($CD45RA^-/CD45RO^+$) in the probiotic group at birth indicating a reduced level of antigen experience in the neonate at birth in probiotic exposed neonates compared to the placebo group. Perinatal probiotic supplementation might therefore reduce the risk of antigenic challenge at birth, while increasing the naïve T cell pool on guard to respond to antigenic challenge. In the current study, perinatal probiotic supplementation appears to potentiate the naïve T cell repertoire at birth.

Increases in allergen-induced Th1 cytokine production by atopic children is associated with expansion of an allergen-specific memory CD45RO T cell population, since memory cells produce 10-fold more IFN-γ than naive T cells (Smart and Kemp 2002). It was therefore noteworthy that the memory CD45RO T cell population was reduced at birth in probiotic exposed neonates compared to placebo group. Under appropriate stimulation, naïve $CD4^+D45RA^+$ T cells can be phenotypically converted *in vitro* into memory type $CD4^+D45RO^+$ T cells (Early and Reen 1999) and acquires memory cell characteristics such as helper activity for B cells, and production of IL-4 and IFN-α (Akbar *et al.* 1988; Clement *et al.* 1988; Ferrer *et al.* 1992). Furthermore, large pools of antigen-experienced effector memory

CD4 and CD8 (T_{RM} cells) with enhanced effector capabilities persist in uninflamed peripheral organs, such as the lung, skin and gut, providing a first line of defence against pathogens (Clark *et al.* 2006; Wakim *et al.* 2008; Vezys *et al.* 2009; Masopust *et al.* 2010; Purwar *et al.* 2011; Sheridan and Lefrancois 2011).Therefore, increases in the naïve CD45RA pool in the probiotic group may reflect an immune signature in which atopic or infectious responses are reduced and the integrity of T cell immunity is strengthened in the neonate due to the increases in the naïve T cell repertoire with potential effector memory cell capabilities. This result was further supported by the reduced CD45RO T cell pool in neonatal peripheral blood in the probiotic group suggesting a reduction in antigenic memory responses in the probiotic group compared to the placebo group. This result further supported the previous observation that $CD3^+$ T cell activity in the neonate was reduced in the probiotic group at 6 months compared to the placebo group.

6.4.6 Effects of perinatal probiotic supplementation on lymphocyte subsets

NK cells have a role in establishing blood supply to the fetoplacental unit (King and Loke 1991) and a key role of NK cells at this site is postulated to be the provision of necessary cytokines such as GM-CSF, TNF-α, IFN-γ, TGF-β and LIF, with minimal impact on cytotoxicity. Interestingly, the percentage of lymphocytes that were NK cells was significantly lower in the probiotic versus placebo group at birth ($p = 0.005$) in the current study and may indicate a need to limit immune toxicity at the neonatal stage. However this result was somewhat unexpected since dietary probiotic supplementation has been linked to an enhancement of the peripheral blood circulating levels of NK cells and enhancement of tumouricidal NK cell activity in healthy elderly subjects (King and Loke 1991; Gill *et al.* 2001).

6.4.7 Regulatory T cells

$CD4^+$ regulatory T cells are particularly abundant and potent at birth and there is much interest in their contribution to the etiology and onset of allergic disease, in early life (Fontenot *et al.* 2003; Godfrey *et al.* 2005; Liu *et al.* 2006). A functional analysis of this subset was beyond the scope of this study but a phenotypic analysis was included in the flow cytometry panel. The probiotic group at birth but not at 6 months of age had significantly fewer $CD4^+CD25^+$ T cells than the placebo group.

This result was unexpected because it is has been demonstrated that neonates at high atopic risk generated fewer $CD4^+CD25^+$ (Schaub *et al.* 2008).

Although the use of extracellular markers is commonly used to identify T reg phenotypes to characterize changes in Treg numbers during childhood caution must be warranted when identifying Tregs based on expression of only CD4 and CD25 as in some circumstances activated T cells adopt a similar phenotype. Therefore possible perturbations in the Treg repertoire should be confirmed by expression levels of the transcription factor foxP3

6.4.8 Gut homing T cells

Since immunological effects of probiotic bacteria are likely mediated via the gut; to determine whether perinatal probiotic supplementation reinforces the integrity of intestinal T cell immunity the analysis of putative gut homing T cells in the circulation was considered as a surrogate of gastrointestinal immunological activity. Maternal supplementation with probiotics was associated with reduction of $CD8^+$ gut homing T cells at birth but not at 6 months of age. Therefore probiotics appeared to induce a reduction in the cytotoxic T cell response at birth but not at 6 months of age.

6.4.9 Dendritic cells

In studies of perinatal probiotic supplementation for the prevention of allergic disease little attention has been paid to the ratio of pDCs and mDCs and the subsequent risk of clinical atopic disease (Upham *et al.* 2004). We had hypothesized that perinatal probiotic supplementation might increase the circulating pDCs levels, an event cited as being protective against atopic outcomes. However, no significant differences in the total DCs or either of the DCs subsets (mDCs and pDCs) at birth or 6 months in those taking probiotics was observed. This result was unexpected especially in the light of the results of other authors. reporting that blood pDCs are less frequent in atopic or asthmatic children compared with healthy subjects (Hagendorens *et al.* 2003). Similarly, in a large cohort of children with severe respiratory syncytial virus–induced bronchiolitis followed up until the age of 6 years who had asthma, the frequency of blood pDCs at 6 years was less than seen in children who did not (Silver *et al.* 2009). Furthermore, (Upham *et al.* 2009) assessed the relationship between the relative frequencies of circulating DC subsets in early childhood by flow cytometry

and discovered that pDCs were significantly less frequent in children who subsequently had an asthma diagnosis by age 5 years compared with children with no asthma diagnosis. They found that the frequency of circulating pDCs was inversely correlated to the cumulative rate of asthma diagnosed by a doctor. Although the frequency of pDCs appeared unrelated to the incidence of allergic sensitization, parent-reported asthma diagnosis by age 5 years was also associated with significantly fewer pDCs during infancy. The authors concluded that having fewer pDCs during infancy was a principal risk factor for asthma-related outcomes by the age of 5 years, whereas having more pDCs during infancy was associated with relative protection against asthma-related outcomes (Upham *et al.* 2009). In the light of the result of other authors it is plausible that the birth to 6 month time period may be too short to observe the immunological manifestation of atopic disorder at the level of the DC subsets.

6.4.10 Limitation of the current study

There are a number of mechanisms by which the frequency of lymphocyte subsets might be reduced in some children, including impaired differentiation from bone marrow precursors, poor cell survival, and increased infection related homing to peripheral tissues. However to our knowledge there were no adverse events as a consequence of probiotic supplementation (Allen *et al.* 2010).

7.1 Background

There are now a number of publications detailing the effects of probiotic supplementation in early life on the development of allergy and allergic disease. These fall broadly into two categories: those in which supplementation to the pregnant woman for the last few weeks of pregnancy and then to the child from birth sometime up to 2 years of age was made; and those in which supplementation of the infant only was made. Studies found to have a beneficial effect fall into the category in which supplementation of the pregnant woman, typically from around 36 weeks of gestation until birth, was included (Kim *et al.* 2009). This raises the possibility that it is supplementation of the pregnant woman and the downstream effects of this on maternal immune function and immune function at the materno-fetal interface that are critical to any biological effect exerted by probiotics on the offspring. The impact of probiotic supplementation on the quality of passively transferred immunity in breast milk also needs to be taken into account.

To gain an appreciation for the likely immune responses elicited by probiotic exposure of pregnant women, understanding the nature of maternal immunity during pregnancy is paramount. Two unique immunological stories unfold during pregnancy. Firstly, a foetal allograft develops in an apparently sterile environment and acquires the essential framework of an immune system enabling it to mount an adequate, albeit immature, immune response. Secondly, a combination of foetal and maternal immunological factors conspires at the maternal-foetal interface to allow, and perhaps even encourage, growth of the semi-allogeneic foetus (Figure 6.1). Indeed emerging evidence suggests that the immunological recognition of pregnancy is paramount to the maintenance and success of gestation (Aagaard-Tillery *et al.* 2006).

A number of theories have been suggested to explain the immunological survival of the foetus within the self/non-self-paradigm encompassing notions of: (1) Anatomic separation of maternal and foetal tissues. (2) Antigenic immaturity of the foetus. (3) An attenuated maternal immune system and (4) the immune privileged site of the uterus. There is a vast literature relating to attempts by investigators to identify

mechanisms explaining the ability of the immune-competent mother to tolerate the semi-allogeneic foetus within this conceptual framework. These strategies include:
1. Preferential expression of non-classical MHC Class I molecules HLA-E, F and G which are less polymorphic than the classical HLA class I molecules. All three of these are expressed on the extravillous trophoblast and have a role in modulating NK cell activity.
2. NK cells which have a role in controlling implantation and establishing blood supply to the fetoplacental unit (King and Loke 1991). There is an accumulation of NK cells in the maternal decidua in the first and second trimester with a reduction in the third trimester (Bulmer *et al.* 1991; Williams *et al.* 2009). A key role of NK cells at this site is postulated to be the provision of necessary cytokines such as GM-CSF, TNF-α, IFN-γ, TGF-β and LIF, with minimal impact on cytotoxicity.
3. Indoleamine 2, 3,-dioxygenase (IDO) is the first and rate-limiting enzyme in tryptophan degradation. IDO degrades the indole moiety of tryptophan, serotonin and melatonin and initiates the production of neuroactive and immunoregulatory metabolites known as kynurenines. This leads to local depletion of tryptophan and increased kynurenines both of which affect T cell proliferation and survival (von Bubnoff *et al.* 2003; Terness *et al.* 2007; Curti *et al.* 2009). Treatment of pregnant mice with a pharmacologic inhibitor of IDO induced maternal T cell-mediated rejection of allogeneic but not syngeneic fetuses (Munn *et al.* 1998).
4. Complement regulation during pregnancy is critical to preventing maternal-mediated damage of the placenta and foetus. Regulators of complement activation - decay accelerating factor (DAF; CD55), CD59, and membrane co-factor protein (MCP; CD46) - are expressed extensively in the human placenta including the syncytiotrophoblast of the placenta that are in direct contact with maternal blood (Girardi *et al.* 2006).

However the strategies most relevant to the work presented in this thesis relate to changes in adaptive immunity reflected by variation in T cell populations and the cytokines they produce, and the innate immune response.

7.1.1 T cell subsets

Although relative deficiencies of Th1 immune function in early life are reported as an explanation for persistent Th2 allergic responses, the inhibition of Th1 function is

important for fetal survival in pregnancy (Prescott, S. and A. Nowak-Wegrzyn 2012). The fetus is a passive contributor to this process with weak Th1 function caused by immaturity, principally imparted by epigenetic mechanisms that alter methylation patterns and gene expression at the IFN-γ promoter in effector $CD4^+$ T cells (North 2011).

The concept of a Th2 biased immune response during pregnancy has dominated the reproductive immunology literature for around 20 years (Lin *et al.* 1993; Wegmann *et al.* 1993; Piccinni *et al.* 2000). However, mice lacking expression of the main Th2 cytokines IL-4, IL-5, IL-9, and IL-13 (i.e. quadruple knockouts) do not have fertility problems (no differences in litter sizes and all offspring healthy) indicating that these cytokines are not essential for foetal survival even during allogeneic pregnancy (Fallon *et al.* 2002). Initial studies focused on the T cells themselves but there are very few T cells at the materno-fetal interface and the paradigm has evolved to encompass type 1 and type 2 cytokines irrespective of their cell source which can include placenta and foetal membranes (Jones *et al.* 1997). Recognition of temporal and spatial differences in the dominance of either type 1 or type 2 cytokines has now emerged and there is now an appreciation of the need for balanced type 1/type 2 responses depending on the tissue site and the stage of pregnancy. Other T cell subsets, namely regulatory T cells and Th17 cells are also of interest, and it has been suggested that homeostasis between regulatory and pro-inflammatory $CD4^+$ T cell subsets might be pivotal for maternal immune tolerance of foetal antigen (Gustafsson *et al.* 2008).

7.1.2 Innate immunity

Innate immunity/inflammation is now considered to have a physiological role in implantation, the maintenance of pregnancy and parturition (Ashkar *et al.* 2000; Croy *et al.* 2002; Patni *et al.* 2007). There is extensive evidence of a heightened systemic inflammatory response in pregnant women (Sacks *et al.* 1999). For example, increased white blood cell count and increased levels of C-reactive protein and other acute phase proteins (Miller 2009). This might be balanced by increased circulating levels of anti-inflammatory molecules such as TGF-β1 (Power *et al.* 2002) and IL-4 (Zhao *et al.* 2009). The enhanced systemic inflammatory response during pregnancy has been postulated to be mediated by placenta-derived microparticles that get into the maternal circulation and are postulated to drive systemic inflammation in normal healthy pregnant women (Sargent *et al.* 2006; Redman and Sargent 2008).

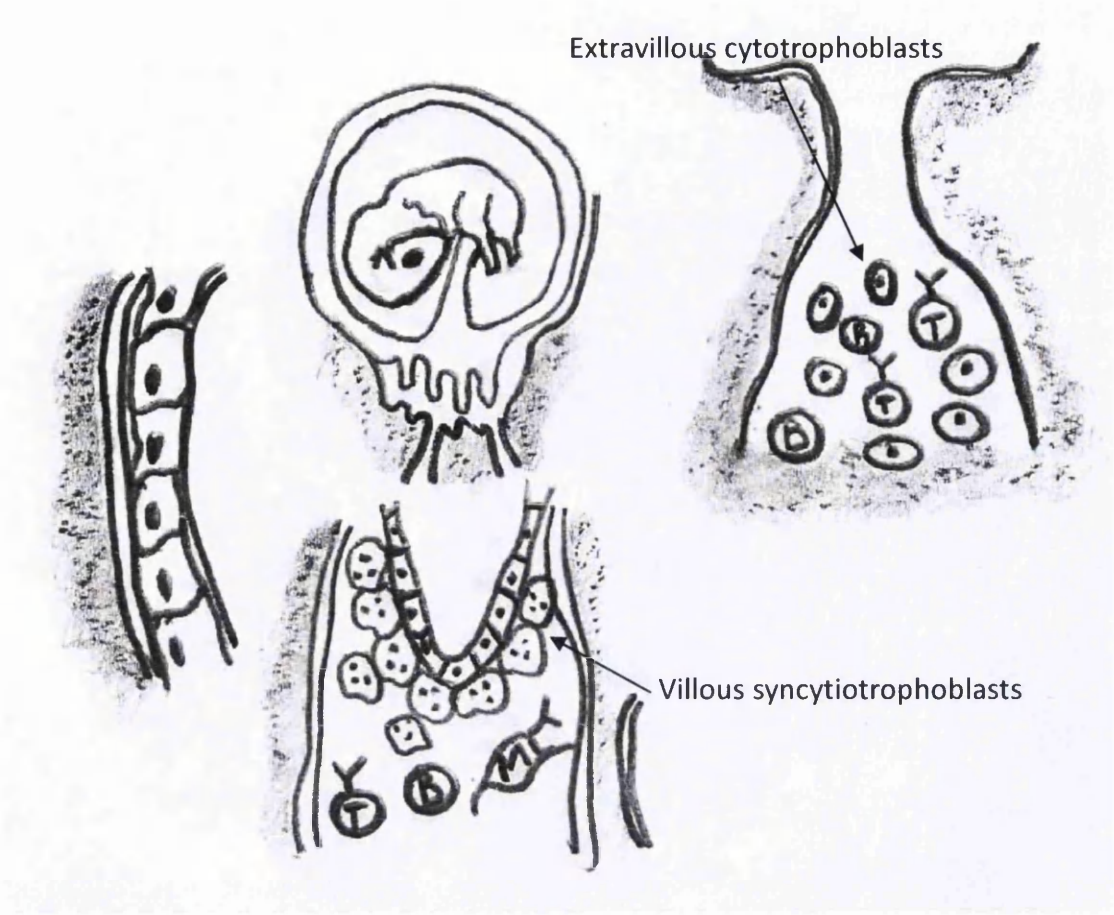


Figure 7.1: Diagram of materno-fetal interface

The placenta forms the principal foetal tissue in direct contact with the maternal immune system. The primary cell type infiltrating the placental villus which is bathed in circulating maternal blood and its constituent haematopoietic cells are the villous syncytiotrophoblast cells which represents the chief cellular component interactive with the maternal immune system. Extravillous cytotrophoblasts form columns of cells that invade into the maternal deciduas. Diagram redrawn and adapted with permission from (Aagaard-Tillery *et al.* 2006).

7.1.3 Breast milk

Progress in research on human lactation and breast milk has advanced our knowledge about the significance of breast milk for the infant (Levy 2007; Lonnerdal 2010). Several growth factors and cytokines such as TGF-β1 and IL-10 are present in breast milk and their capacity to persist in the infant gut and exert their activities is postulated to influence maturation of immune function and the development of oral tolerance (Peroni *et al.* 2010). Recent findings show that specific strains of bacteria are present in breast milk and act as probiotics during early colonisation of the

infant's intestine and that human milk oligosaccharides (prebiotics) are specific substrates for probiotic strains. Amongst the bacterial groups found in breast milk, lactobacilli constitute an important group. One of the *Lactobacillus* strains isolated from breast milk with an important probiotic potential is *Lactobacillus salivarius* CECT5713 (Martin *et al.* 2006) this strain is endowed with a powerful anti-bacterial properties able to strongly protect mice from *Salmonella* infection (Olivares *et al.* 2006). Moreover, *Lactobacillus salivarius* modulates the immune response in animal models showing an anti-inflammatory character able to prevent colonic inflammation in a rat model of colitis (Peran *et al.* 2005; Diaz-Ropero *et al.* 2007).

7.1.4 Need for the study

Perinatal probiotic supplementation, which includes supplementation of the pregnant woman, is an emerging therapeutic feature in contemporary paediatric immunology to strategically facilitate improvement in the quality of passively transferred immunity and newborn microbial populations. Although probiotics have a long record of safe use (Allen *et al.* 2010) and have been administered widely to pregnant women in probiotic birth cohort supplemental studies, there is a paucity of research both *in vitro* and *in vivo* characterising the immunomodulatory effects of probiotic supplementation on immune parameters during pregnancy. Probiotic supplementation of the pregnant woman is postulated to induce beneficial gastro-immune equilibrium within the offspring; characterised by establishment of a favourable microfloral foundation in the neonatal gut ultimately offering protection against intestinal barrier microbial aberrancies and immune dysfunctions by attenuating the detrimental effects of a microbiota lacking in beneficial microorganisms (Barouei *et al.* 2009). While research suggests that such a pathway may exist, no studies have directly investigated this relationship in terms of the immunomodulatory role of probiotics during pregnancy.

This *in vitro* study attempts to link probiotic supplemental approaches and their possible prophylactic outcomes on reproductive immunology. Using a similar *in vitro* model as in chapter 3, the effects of the study consortium on cytokine responses in peripheral blood mononuclear cells prepared from pregnant and non-pregnant women was investigated.

Hypothesis:

The Th1 response to the consortium will be attenuated in pregnant woman compared to the non-pregnant woman, while the IL-10 immunosupressive response will be upregaluated in the pregnant woman.

7.2 Rationale and experimental approach

7.2.1 Samples and subjects

Peripheral blood (10 – 15ml) from healthy pregnant women undergoing pre-anaesthetic check prior to elective caesarean section at term and healthy non-pregnant women was collected into sodium heparin as anticoagulant. For ease and clarity, unless stated otherwise, the prefix pPBMCs indicates mononuclear cells obtained from peripheral blood of pregnant women and nPBMCs mononuclear cells from peripheral blood of non-pregnant women.

7.2.2 Co-culture of pPBMCs and nPBMCs with the study consortium

PBMCs were isolated by density gradient centrifugation and used as follows:

1. Cells (1 x 10^6/ml) were co-cultured alone or in the presence of a dose course of 10^6, 10^5, 10^4, 10^3 and 10^2 CFUs/ml of the study consortium, as described in section 2.1.4 Cell/bacteria free supernatants were harvested and stored at -20° C until analysis using ELISAs.
2. Cells (1×10^6/ml) were co-cultured with 10^6 CFUs/ml of the study consortium for 24 hours and then cultures left untreated or treated with PHA (1μg/ml) or SEB (200ng/ml) for a further 24 hours. Cell/bacteria free supernatants were harvested by centrifugation and stored at -20°C until analysis using ELISAs.

7.2.3 Baseline levels of mononuclear leukocyte subpopulations in pPBMCs and nPBMCs

PBMCs prepared from blood of pregnant and non-pregnant donors were subject to flow cytometry to compare the leukocyte composition. This was intended to provide insight into any differences in cytokine responses seen between the two groups. The cell types studied were: $CD14^+$ monocytes, $CD19^+$ B cells, CD16/CD56 expressing

NK cells, $CD3^+/CD4^+$ T helper cells and $CD3^+/CD8^+$ cytotoxic T cells. After preparation of MNCs by density gradient centrifugation, 0.5 x 10^6 cells were incubated with fluorochrome conjugated antibodies for 30 minutes on ice in the dark. Cells were then washed by centrifugation in FACS buffer, supernatants discarded and the cell pellet re-suspended in 200µl FACS fix prior to acquisition within 24 hours.

7.2.4 Statistical analysis

The student's t test was employed to determine whether: (1) cytokine responses from pPBMCs and nPBMCs in response to a dose course of the study consortium were different when compared to the unstimulated mononuclear control; and (2) if consortium induced TNF-α, IL-12p70, IL-10 and TGF-β1 responses differed beteween pPBMC compared to nPBMCs.

7.3 Results

7.3.1 Effects of the study consortium on cytokine responses from PBMCs isolated from pregnant and non-pregnant women

Consistent with the data shown in chapter 3, TNF-α, IL-12p70 and IL-10 were produced in response to all doses of the study consortium by all women (Figure 7.2). This response was statistically significant at 10^6 and 10^5 CFUs/ml compared with no bacteria for all three cytokines in both groups of women. Persistence of a significant effect at lower doses differed for each of the cytokines with a significant effect on TNFα persisting even at the lowest dose studied (10^2 CFUs/ml) whereas a significant

effect on IL-10 was lost by 10^4 CFUs/ml for non-pregnant women but not until 10^3 CFUs/ml for pregnant women. Other than the IL-10 response at 10^5 CFUs/ml ($p = 0.04$) and 10^4 CFUs/ml ($p = 0.03$) in which PBMCs from pregnant women gave the higher response, and the higher IL-12p70 response by PBMCs of pregnant women at 10^2 CFUs/ml ($p = 0.03$), there were no differences between the responses by cells from pregnant and non-pregnant donors. TGF-β1 occurs in a latent form and requires activation by acidification and then neutralisation to measure total TGF-β1. TGF-β1 levels after activation did not show any association with the dose of the study consortium used (Figure 7.3).

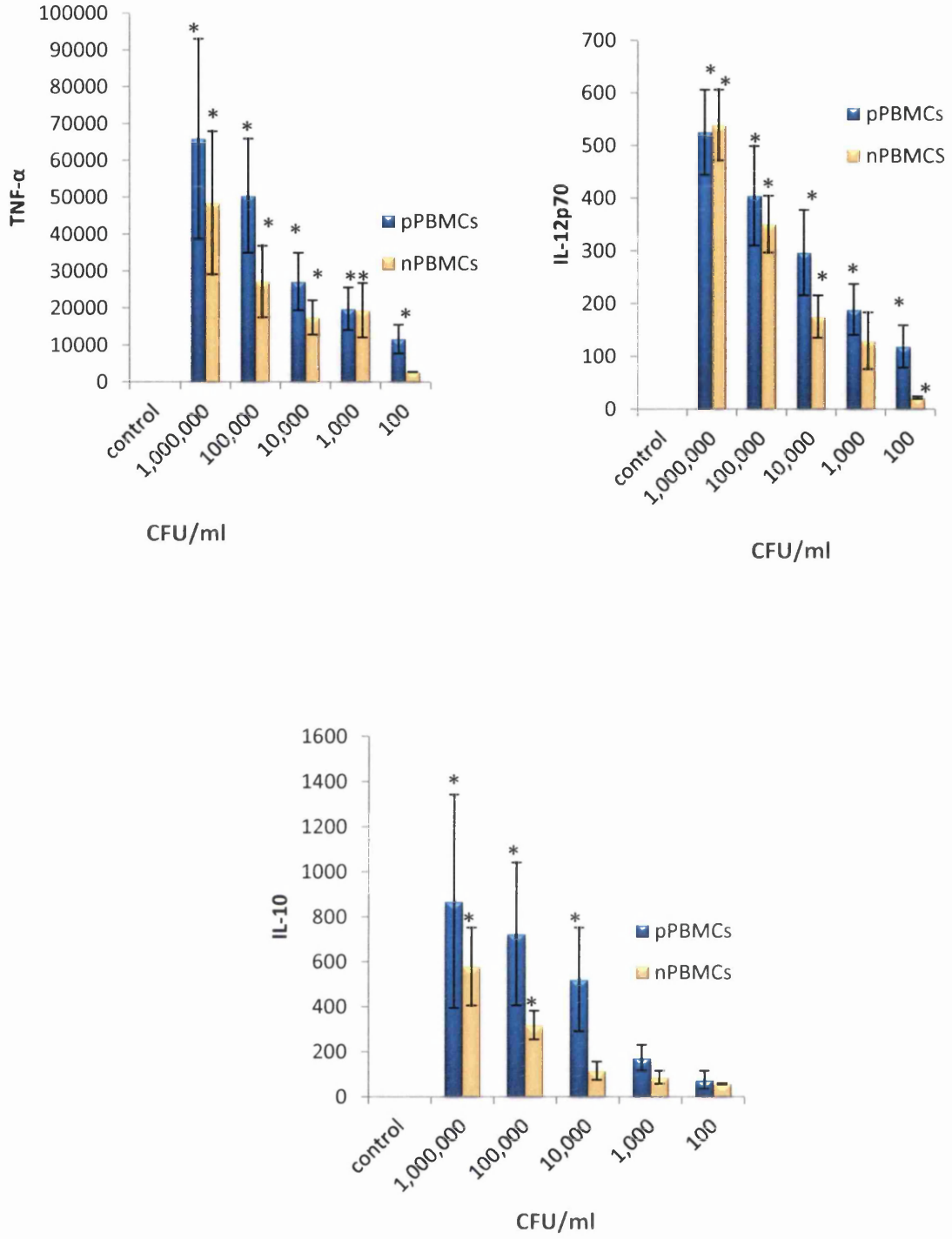


Figure 7.2: Cytokines produced by peripheral blood mononuclear cells of pregnant (pPBMCs; n = 10 – 12) and non-pregnant (nPBMCs; n = 7 – 8) women in response to a dose course of the study consortium. Cell/bacteria free culture supernatants were prepared after 24 hours incubation for measurement of (a) TNF-α, (b) IL-12p70, and (c) IL-10 production (all pg/ml) using specific ELISAs. Results are shown as mean ± SEM. Significant differences ($p < 0.05$) between the response by pPBMCs and nPBMCs compared to the un-stimulated mononuclear cell control is denoted by *.

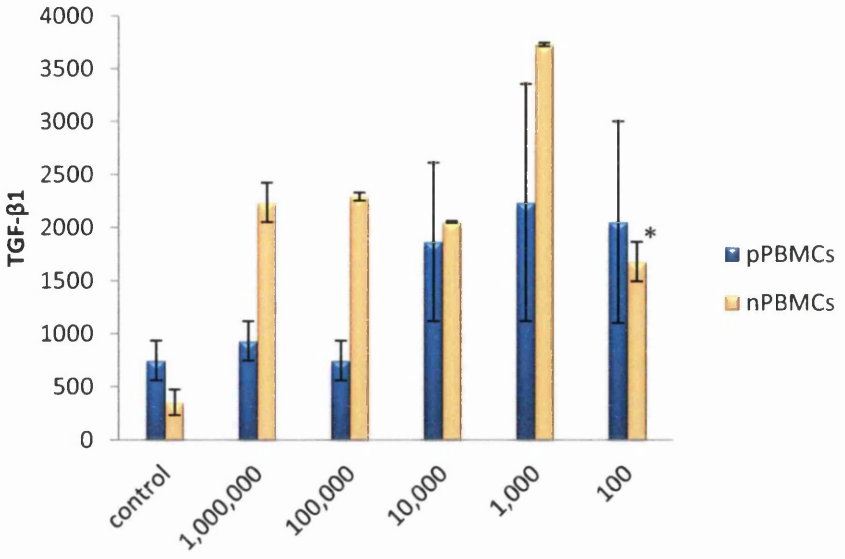
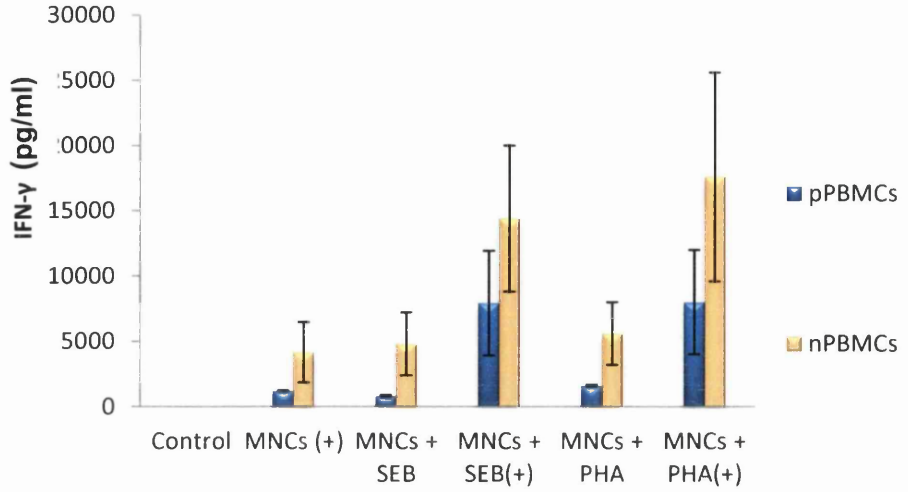


Figure 7.3: TGF-β1 produced by peripheral blood mononuclear cells of pregnant (pPBMCs; n = 12) and non-pregnant (nPBMCs; n = 7) women in response to a dose course of the study consortium.
Cell/bacteria free culture supernatants were prepared after 24 hours incubation for measurement of TGF-β1 after activation (acidification/neutralisation) using a specific ELISA. Results are shown as mean ± SEM. Significant differences ($p < 0.05$) between the response by pPBMCs and nPBMCs compared to the un-stimulated mononuclear cell control is denoted by *.

7.3.2 Immunomodulatory effects of the study consortium upon polyclonally stimulated mononuclear cells isolated from pregnant and non-pregnant women.

For these experiments, PBMCs were cultured with 10^6 CFUs/ml of the study consortium (denoted with the prefix (+)) for 24 hours prior to stimulation with PHA or SEB for a further 24 hours. Both PHA and SEB increased IL-17 levels in supernatants of nPBMCs and pPBMCs. PHA-stimulated IL-17 was reduced in PBMCs from pregnant versus non pregnant women ($p = 0.046$) and probiotics induced a significant increase in PHA-induced IL-17 in PBMCs from pregnant but not non pregnant women ($p = 0.002$).

(a) IFN-γ



(b) IL-17

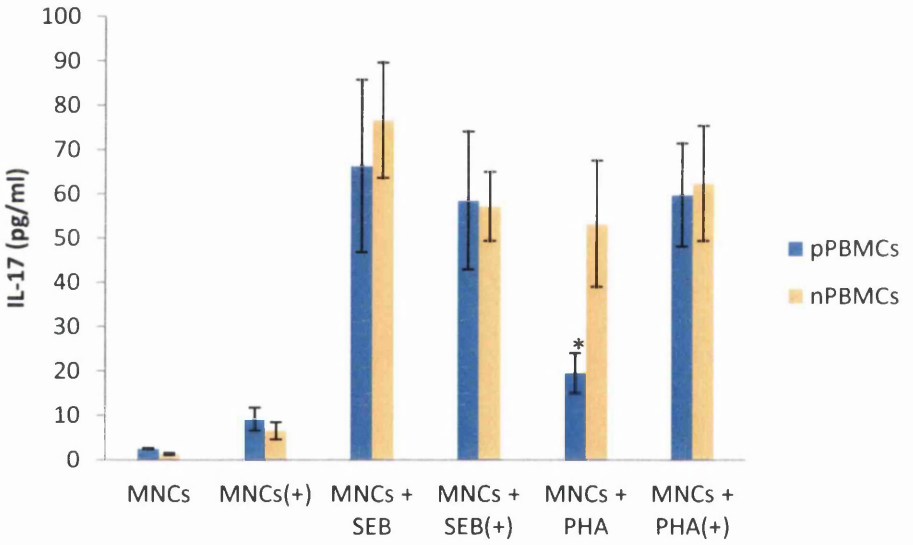


Figure 7.4: Cytokines produced by peripheral blood mononuclear cells of pregnant (pPBMCs) n = 10 and non-pregnant (nPBMCs) n = 8 women in response to 10^6 CFUs of the study consortium alone or after addition of PHA or SEB. Cell/bacteria free culture supernatants were prepared after 24 hours incubation with/without probiotic and a further 24 hours with/without PHA or SEB for measurement of (a) IFNγ, and (b) IL-17 (all pg/ml) using specific ELISAs. The student t test was employed to determine whether there was a statistically significant difference in mononuclear cell response between (1) consortium and the control. (2) SEB or PHA stimulated mononuclear cells versus the control and (3) SEB or PHA stimulated mononuclear cells alone versus SEB or PHA stimulated mononuclear cells in the presence of the consortium. Details of p-values < 0.05 are denoted on the graphs with an *

7.3.3 Basal levels of mononuclear leukocyte subpopulations in healthy pregnant and non-pregnant women

The cellular content of PBMC preparations from pregnant and non-pregnant women was monitored. The gating strategy used is shown in Figure 7.5. A gate was first drawn around mononuclear cells based on their characteristic forward and side scatter profiles (Figure 7.5a). Events within this gate were re-drawn on the basis of CD19 versus CD14 expression to identify B cells and monocytes, respectively (Figure 7.5b), and again on the basis of CD3 and CD16/56 expression to identify T cells and NK cells, respectively (Figure 7.5c). The isotype control is also shown and this facilitated the placing of quadrants on the dot plots (Figure 6.5d).

Pregnant women were found to have significantly fewer total $CD3^+$ T cells in the PBMC preparation than non-pregnant women ($p = 0.00027$; Figure 6.6), but they had higher proportion of B cells ($p = 0.023$). Monocytes tended to be more abundant in PBMCs from pregnant women but this did not reach significance ($p = 0.0892$); NK cells tended to be lower in PBMCs prepared from peripheral blood of pregnant women but this did not reach statistical significance. On combining $CD3^+$ T cells, $CD19^+$ B cells and $CD16^+CD56^+$ NK cells as total lymphocytes, and comparing to whole blood available for these women (Figure 7.7), lymphocytes were less abundant in samples from pregnant women in both MNC preparations and whole blood ($p = 0.00003$ and 0.07741, respectively).

Consideration of the relative abundance of the two main T cell subsets was then made in the mononuclear cell preparations. To identify these subsets – $CD3^+CD4^+$ T helper cells and $CD3^+CD8^+$ cytotoxic T cells – a gate was drawn around $CD3^+$ cells (Figure 7.8a) and expression of CD4 of CD8 by these $CD3^+$ cells then investigated (Figure 7.8b). There was no difference between the relative abundance of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in pregnant and non-pregnant donors (Figure 7.9).

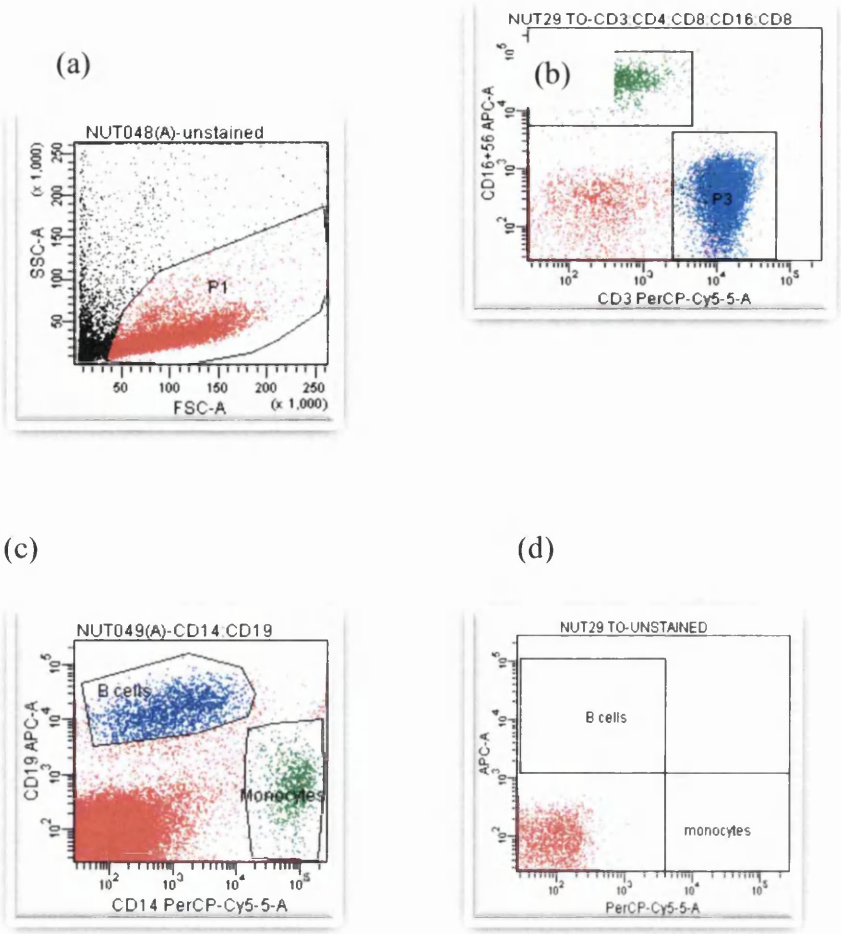


Figure 7.5: Gating strategy for identification of mononuclear leukocyte subsets
Mononuclear cells were first identified by their characteristic side scatter (SSC) and forward scatter (FSC) profiles and a gate drawn around these cells (a). Expression of (b) CD14 (anti-CD14:PE-Cy5.5) and CD19 (anti-CD19:APC), and (c) CD3 (anti-CD3:PE-Cy5.5) and CD16/56 (both antibodies conjugated to APC) were determined in separate tubes. An isotype control is shown in (d).

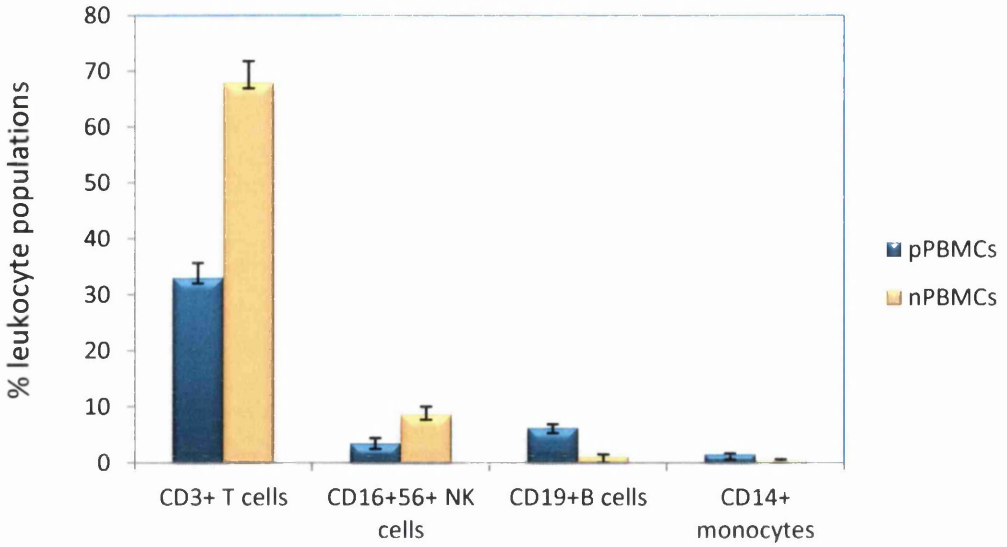


Figure 7.6: Basal levels of $CD3^+$ T cell, $CD16^+/CD56^+$ NK cells, $CD19^+$ B cells and monocytes T cell , B cell and NK cell population levels present in peripheral blood mononuclear cells from pregnant (n = 5) and non-pregnant (n = 4) women.

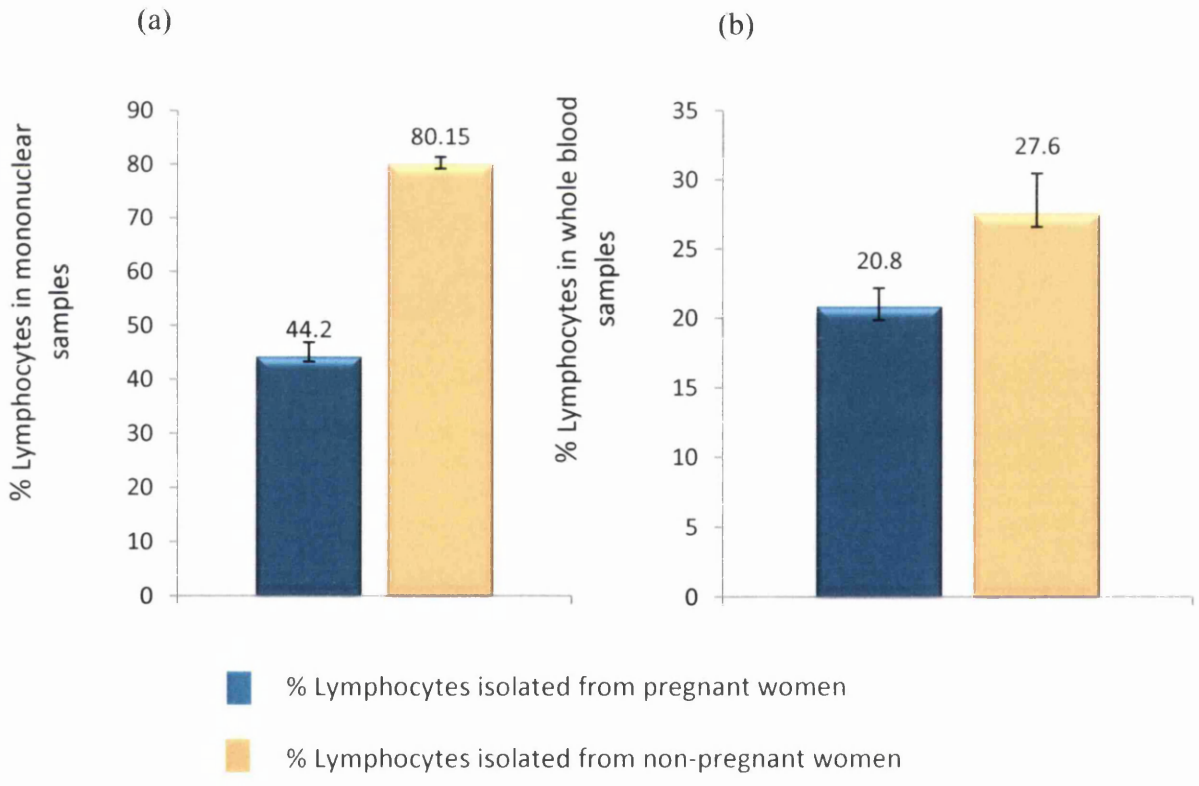


Figure 7.7: Relative abundance of lymphocytes in (a) mononuclear cells, and (b) whole blood from pregnant (n = 5) versus non-pregnant (n = 4) women.
Lymphocytes in PBMC preparations were determined using flow cytometry and in whole blood using automated haematology analysis.

(a)

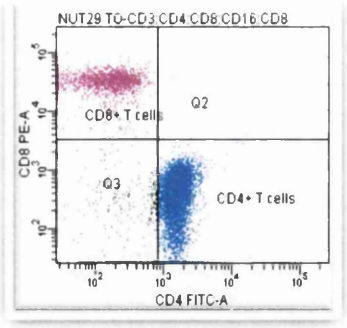


Figure 7.8: Gating strategy used to identify the two major subsets of T cells.
A gate was drawn around $CD3^+$ cells, and then expression of (a) CD4 (anti-CD4:FITC) and CD8 (anti-CD8:PE) by these cells investigated.

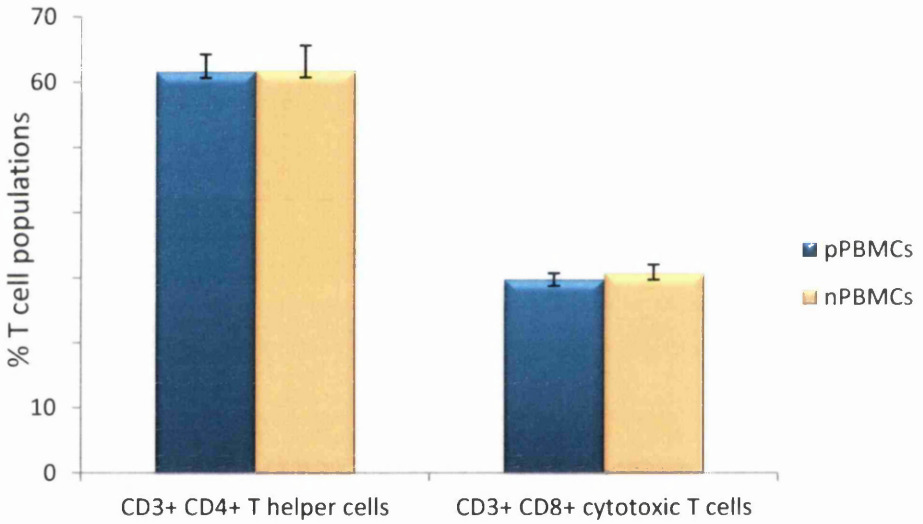


Figure 7.9: Relative abundance of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in total $CD3^+$ population in PBMCs of pregnant versus non-pregnant donors.
$CD3^+$ cells were identified using flow cytometry, a gate was drawn around these and then the cells positive for CD4 or CD8 identified

7.4 Discussion

7.4.1 Role of the study consortium on the induction of inflammatory responses from mononuclear cells extracted from both pregnant and non-pregnant women

This *in vitro* study served to determine whether the immunomodulatory roles of probiotic supplementation during pregnancy could potentially impact positively on foetal immunology. While it remains controversial whether true suppression of the maternal immune response exists, multiple lines of evidence suggest that soluble secreted factors in the form of cytokines having immunosuppressive or immunomodulatory capacity are associated with implantation and foetal survival. Focus on the enigma of how the foetal semi-allograft avoids immunological attack by the immune system of the pregnant mother has led investigators to assume that a maternal response to foetal antigens will be harmful to the foetus. This paradigm is known as immunodystrophism. Moreover inhibitory effects of the maternal immune system need not be deleterious to the foetus and may serve to limit the extent of trophoblast invasion which occurs during the first 20 weeks of normal pregnancy (Whitley *et al.* 2007). A range of cytokines appear to be involved during pregnancy, collectively having roles at the maternal-foetal interface.

Consistent with results in Chapter 3, production of the pro-inflammatory cytokines IL-12p70 and TNF-α by mononuclear cells occurred in a dose dependent manner in response to the consortium and was greatest at the highest consortium doses of 10^6 and 10^5 CFUs/ml, for both study groups. An attenuation of the IL-12p70 response occurred on decreasing consortium concentration. Interestingly consortium concentrations ranging from 10^5 CFUs to 10^2 CFUs induced greater IL-12p70 responses from pPBMCs compared to nPBMCs and this was most apparent at the lower consortium concentrations and the difference was statistically significant at a doses of 10^2 CFUs/ml (p value =0.03) suggesting that pPBMCs at lower consortium doses have a greater capacity than nPBMCs to produce the inflammatory cytokine IL-12p70. Whether the consortium can induce low or high concentrations of IL-12p70 during pregnancy *in vivo* and whether this places immunological constraints or benefits on reproductive immunology still needs further clarification.

Interestingly, TNF-α production by pPBMCs in response to the consortium at all doses analysed compared to nPBMCs was superior although not statistically significant. Since TNF-α is a marker of heightened immunological activity and a cytokine that induces growth and differentiation it seems reasonable that its production in response to the consortium would be greater in pregnant versus non pregnant women to reflect the heightened state of growth and development occurring during pregnancy to perhaps regulate excessive trophblast cellular invasion. Trophoblast cells display a unique capability marked by a physiological invasion of the surrounding tissue. Although their invasive nature bears resemblance to the behaviour of tumors the imposing difference is that under normal physiological conditions trophoblast invasion is temporally and spatially controlled in contrast to the unlimited invasive nature of tumors and initiates immediately after embryo implantation into the endometrium (Fitzgerald *et al.* 2008). Parallel to tumours, trophoblasts secrete proteases, such as matrix metalloproteinases, which dissolve the extracellular matrix and the surrounding tissue thereby facilitating trophoblasts invasion. The invasive capacities of trophoblasts are positively and negatively regulated by numerous cytokines including leukaemia inhibitory factor, IL-6 however their roles are beyond the scope of this discussion; for a good review see Fitzgerald *et al.* (2008). The role of TNF-α will be particularly considered for the benefit of the discussion. A commonly appreciated notion is the paradigm of immunodystrophism which suggests that the maternal immune response to foetal antigens is harmful to the foetus (Anderson *et al.* 1991). However, inhibitory effects of the immune system need not be deleterious to the foetus and TNF-α induction has been demonstrated to limit the extent of trophoblast invasion during normal pregnancy. Histological evaluation of ectopic pregnancies (Paterson and Grant 1975) in which trophoblast is dramatically more invasive and destructive, suggests that local factors in the decidua and/or endometrium inhibit trophoblast invasiveness. While it remains controversial whether immunological suppression of the maternal immune response occurs *in vivo*, multiple lines of evidence suggest that soluble factors associated with implantation and foetal survival are secreted and collectively have immunosuppressive or immunomodulatory capacity.

The soluble factors TNF-α, IFN-γ and IFN-α/β can inhibit *in vitro* rodent trophoblast cultures (Hunt *et al.* 1990) and similar cytokines, including granulocyte colony stimulating factor ., can restrict the growth of the human choriocarcinoma cell line, JEG-3 (Berkowitz *et al.* 1988). This restriction may be due to an inhibition of

trophoblast cell DNA synthesis by the cytokines (Hunt, Lessin *et al.* 1989) It is uncertain whether cytokines have direct toxicity against trophoblasts. Although one group demonstrated that trophoblast monolayers can be disrupted by TNF-α or IFN-γ (Yui J *et al.* 1993), another found that TNF-α did not kill trophoblast cells *in vitro* (Drake and JR 1990). Additionally the study consortium alone was able to stimulate significant amounts of IFN-γ production from pPBMCs and nPBMCs in comparison to the un-stimulated controls. IFN-γ production from pPBMCs in response to the consortium was statistically significant. Although the consortium also stimulated IFN-γ production from nPBMCs, the result was not statistically significant, and there were no significant differences between IFN-γ responses of pPBMCs and nPBMCs.

Although physiological levels of these cytokines are present during normal pregnancy, harmful levels may be produced in certain pathological conditions such as infection and hypoxia and it is important to monitor whether probiotic supplementation during pregnancy alters physiologically levels of TNF-α and IFN-γ *in vivo*. A point of note is that systemic administration of TNF-α (Silen *et al.* 1989; Chaouat *et al.* 1990; Silver *et al.* 1994) and IFN-γ all result in pregnancy loss in murine models (Chaouat *et al.* 1990).

7.4.2 Role of the study consortium on the induction of anti-inflammatory responses from mononuclear cells extracted from both pregnant and non-pregnant women

The anti-inflammatory cytokine, IL-10 included in this study represented a cytokine that supports suppressive and regulatory immune responses during normal pregnancy (Thaxton and Sharma 2010). In the present study, it was discovered that the strongest consortium dose of 10^6 CFUs induce the greatest IL-10 response from PBMCs isolated from both healthy pregnant and non-pregnant women. A dilution of this effect was observed with decreasing consortium concentration and the IL-10 response appeared to plateau at doses between 10^4 and 10^2 CFUs in both pregnant and non-pregnant women.

In marked contrast, the IL-10 response by PBMCs from pregnant women was significantly stronger in comparison to responses observed by PBMCs from healthy women and this was apparent at consortium does of 10^5 and 10^4. In this scenario consortium concentrations of 10^6, 10^5 and 10^4 induced the greatest IL-10 responses (P

= 0.026, 0.07 and 0.013 respectively) although the response began to decline at the lower doses of 10^3 and 10^2 this decline was not statistically significant. The immunological significance of this result is exemplified when considered in the context of the physiological role of IL-10 during pregnancy.

IL-10 is a facilitator of successful pregnancy, both as an immune suppressive agent and a mediator of cross talk between the placenta and the decidua. The role of IL-10 during pregnancy as a suppressor of active maternal immunity to allow acceptance of the foetal allograft is a widely accepted paradigm. For instance IL-10 levels increase markedly in women during early pregnancy and remain elevated well into the third trimester immediately prior to onset of labour (Thaxton and Sharma 2010). Moreover, secretion of IL-10 by a diverse set of maternal and foetal cells inclusive of Trophoblasts, Uterine NK cells, monocytes and Treg has proven to aid in the orchestration of normal processes of pregnancy. However it is unknown whether probiotic bacteria and/or their DNA transcripts occurring within the placenta are interactive with maternal and foetal cells to influence IL-10 production. The observation that IL-10 was increased more preferentially in pregnant women compared to non-pregnant women correlates with the necessity for regulated IL-10 producing during normal pregnancy.

7.4.3 The immunomodulatory effect of a consortium of potential probiotics on Th1 and Th17 cytokine production as represented by IFN-γ and IL-17 production from

As for IFN-γ, the study consortium alone induced more IL-17 from both pPBMCs and nPBMCs in comparison to the unstimulated controls. Additionally, the consortium potentiated PHA-induced IL-17 production by pPBMCs but otherwise had no effect on induced IL-17 production. The apparent lack of Th1 cytokine production in the form of IFN-γ production from pPBMCs compared to nPBMCs in response to the consortium could be in part explained by a reduced CD3 positive T cell repertoire in pregnant women compared to non-pregnant women. One of the major sources of both IFN-γ in the PBMC preparations used is T cells and these were significantly reduced in the preparation from pregnant subjects. This possibility could be considered in future studies using intra-cellular flow cytometry or ELISPOT. The observation that T cells are reduced in the PBMC preparations was supported by decreased lymphocyte numbers in whole blood from the same donors although in the small number analysed this did not achieve significance.

The maternal immune response changes dramatically during pregnancy along with dimensions of the T cell compartment (Koch and Platt 2007). A number of authors have described declines in both the $CD4^+$ and $CD8^+$ peripheral blood T cell subsets over pregnancy: $CD4^+$ T cells decrease in the 2nd and 3rd trimesters while $CD8^+$ T cells decreased during the third trimester (Tallon *et al.* 1984); $CD8^+$ T cells decreased during the 1st trimester while $CD4^+$ T cells decreased in the 3rd trimester with both populations increasing to baseline levels four months postpartum (Watanabe *et al.* 1997). Current results indicate a statistically significant increase in B cells during pregnancy. This could be explained by oestrogen stimulatory effect on humoral immunity which acts on B cells, thereby increasing IgG and IgA synthesises during pregnancy.

Chapter 8: General discussion

8.1 Role of the study consortium during infancy and pregnancy

Inadequate microbial colonisation of the gut is a key environmental determinant contributing to the expression of IgE-mediated atopic disease. Perinatal probiotic supplementation is therefore postulated to contribute to microbiota mediated immunomodulatory responses, critical in shaping development of hypo-responsiveness to allergens (Isolauri 2004).

In the present study mononuclear cells prepared from umbilical cord blood or adult peripheral blood, co-cultured in the presence of the study consortium *in vitro,* were induced to generate a dose dependent, monocyte mediated release of the pro-inflammatory cytokines TNF-α, IL-6, IL-1β, IL-17, IL-12p70, IFN-γ and the immunosuppressive cytokines IL-10 and TGF-β1. Interestingly CBMCs were significantly impaired in their ability to produce IL-12p70 and IL-10 following incubation with the consortium compared to PBMCs, an observation likely attributable to under-developed mechanisms of Th1 immunity in the newborn (Siegrist 2001; Adkins *et al.* 2004; Marchant and Goldman 2005) and the newborn propensity to respond to antigenic stimuli with a Th2 biased scheme of immunity reminiscent of the *in utero* environment. Data from the present study indicated that the consortium induced a subtle mode of inflammation likely necessary to circumvent over-excessive and deleterious Th1 responses during the early stages of life. Furthermore, the consortium down regulated PHA and SEB induced IL-13 (a key allergy orchestrating cytokine) while potentiating IFN-γ (a key Th1 driving cytokine) responses from both PBMCs and CBMCs. Further interrogation of the study

consortium is necessary to determine whether the consortium induces soluble mediators within the cell culture media also capable of inducing cytokine responses from umbilical mononuclear cells. Furthermore whether the consortium induces monocyte maturation marked by the upregulation of co-stimulatory molecules remains to be resolved in the current study

At the maternal-foetal interface, a range of cytokines are involved during pregnancy. It was therefore imperative to gain an insight into the immunomodulatory roles of probiotic supplementation during pregnancy and the downstream impact on foetal immunology. The current study indicated that production of the pro-inflammatory cytokines IFN-γ, IL-12p70 IL-17, TNF-α and the suppressive cytokine IL-10 by mononuclear cells extracted from both pregnant (pPBMCs) and non-pregnant (nPBMCs) women occurred in a dose dependent manner in response to the study consortium. pPBMCs at lower consortium doses had a significantly greater capacity than nPBMCs to produce the inflammatory cytokine IL-12p70. Interestingly there was a lack IFN-γ production from pPBMCs compared to nPBMCs in response to the consortium, likely attributable to the reduced $CD3^+$ T cell repertoire in pregnant women compared to non-pregnant women.

Although physiological levels of the pro-inflammatory cytokines are present during normal pregnancy, harmful levels occur in pathological conditions such as infection or hypoxia. Under normal conditions the induction of the pro-inflammatory cytokines need not be deleterious to the foetus (Hunt *et al.* 1989). In the present study no adverse effects as a result of perinatal probiotic supplementation were reported (Allen *et al.* 2010). Interestingly, the IL-10 response by PBMCs from pregnant women was significantly stronger in comparison to responses observed by PBMCs from healthy women. The immunological significance of this result is exemplified when considered in the context of a physiological role of IL-10 as a facilitator of a successful pregnancy. Furthermore, maternal immune response changes dramatically during pregnancy along with dimensions of the T and B cell compartments (Koch and Platt 2007). Current results indicated a statistically significant increase in B cells during pregnancy. This could be explained by the oestrogenic effect on humoral immunity which acts on B cells, increasing humoral immunity during pregnancy.

8.2 Probiotics in the prevention of atopic disorder: a randomised controlled trial

Administration of a multi-strain, high-dose probiotic to mothers during late pregnancy and their infants from birth to 6 months of age was not significantly effective as a pre-emptive strategy to prevent eczema in infants predisposed to a sentence of atopic eczema in later life during the PROBAT trial (Allen *et al.* 2014). However probiotic administration was associated with a reduced frequency in sensitivity to food antigens, concomitant with a reduced risk of atopic sensitisation by 2 years of age.

8.3 *Ex vivo* Imunomodulatory role of probiotics during infancy

Whether perinatal probiotic supplementation boosts cytokine function in a way that might represent an attenuation of the symptoms of atopy was studied *ex vivo* by studying the down regulation of the Th2 allergy orchestrating cytokines and the up regulation of the Th1 cytokines by mitogen stimulated mononuclear cells isolated from the blood of atopy predisposed neonates recruited to the PROBAT trial at birth and at 6 months of age. Surprisingly, no statistically significant effect of supplementation on IL-4, IL-10, and IL-13 or IFN-γ production by mitogen stimulated neonatal mononuclear cells was observed. Interestingly, PHA induced *ex vivo* IL-13 responses showed an inverse association with age suggesting a reduction in a key allergy exacerbating cytokine in the PROBAT cohort with age. Whether this observation was dependent or independent of perinatal probiotic supplementation remains unresolved.

8.4 Innate immune response in relation to probiotic versus placebo exposure

The immunogenicity of probiotic bacteria upon interaction with the whole blood cellular components of neonatal immunity (such as the granulocytes) renders them key contributors to the pro- and anti-inflammatory cytokine tone within the neonatal gut and the periphery. To test this hypothesis whole blood cultures were stimulated at birth and 6 months of age from recruits on the PROBAT trial with lipopolysaccharide or peptidoglycan, the ensuing cytokine responses were measured. Perinatal probiotic supplementation significantly increased Th1 IL-12p70 cytokine capacity in the probiotic group in unstimulated samples at birth. Additionally LPS/IFNγ induced IL-12p70 responses by whole blood cultures were also significantly increased in the probiotic group at 6 months of age. Further supporting the notion perinatal probiotic supplementation may enhance maturation of *in vivo*

Th1 responses in probiotic treated groups compared to placebo group, and accelerate maturation of an otherwise attenuated Th1 pro-inflammatory cytokine pathway during the neonatal period. Whether or not the observed Th1 immune responses can be linked to protection against IgE-mediated atopic disorder will be reviewed in the light of the clinical outcomes of the PROBAT trial for future studies. Promisingly Th1 polarising cytokine responses are antagonistic to the up-regulation of IgE mediated Th2 atopic responses *in vivo* and contribute to the polarisation of naive T cell towards the Th1 phenotype. These results provide further support for the application of probiotic bacteria as a strategy to attenuate the risk of IgE-mediated atopic disorder at both the cellular and innate immune level during early infancy.

8.5 Cell counts in relation to probiotic versus placebo exposure employing haematological analysis

Hematopoietic myeloid progenitors are postulated to support recruitment of pro-inflammatory cells to tissues sites prominent in atopic diseases (Gauvreau *et al.* 2009). Furthermore hematopoietic progenitors from umbilical cord blood of high risk genetically predisposed neonates give rise to more eosinophil and basophil progenitors involved in the pathogenesis of atopic disorder (Reece *et al.* 2011). Interestingly perinatal probiotic supplementation only had a transient effect on granulopoesis, affecting only the cord blood eosinophil count, which was reduced at birth. Although this result was indirectly linked with the results of Allen *et al.* (2014) highlighting that perinatal probiotic supplementation is associated with a reduction in atopic sensitisation. Future analysis of the PROBAT study outcomes is required to determine whether this observation correlates to a reduction in atopic disorders in later life, where eosinophilia at the sites of allergic inflammation is a hallmark component. Long-term follow-up to determine the effect of probiotic administration on the allergic march is therefore of utmost importance (Ker *et al.* 2009).

8.6 Cell counts in relation to probiotic versus placebo exposure employing immunophenotypic analysis

A flow cytometric approach was employed to consider in more detail the effects of perinatal probiotic supplementation on key lymphocyte subsets, T cell subsets, B cell subsets and dendritic cell subsets on percentages of key lymphocytic subpopulations involved in atopic disorder. On comparing the probiotic and placebo groups the percentage of $CD4^+$ T cells bearing a naive phenotype ($CD45RA^+/CD45RO^-$) was

significantly elevated in the probiotic group at birth. This was complemented by a significantly reduced percentage of $CD4^+$ T cells expressing a memory phenotype ($CD45RA^-/CD45RO^+$) in the probiotic group at birth suggesting that perinatal probiotic supplementation was able to potentiate the naïve T cell repertoire during the neonatal period. This observation may be reflective of a general probiotic mediated reduction of atopic or infectious responses *in vivo* due to a strengthening of adaptive immunity at the cellular level in the neonate. The reduced CD45RO T cell pool in neonatal peripheral blood in the probiotic group could also be indicative of a reduction in antigenic memory responses in the probiotic group compared to the placebo group imparted by a greater proportion of naïve T lymphocytes with the capacity to differentiate to T lymphocytes with effector function against atopic or infectious responses.

8.7 Further work

Further exploration of the current cohort with analysis of faecal samples and plasma/serum to compare local gastrointestinal and systemic inflammatory responses is necessary to gain further insight into the immunomodulatory role of probiotics in infancy. For instance the specificity of human immune responses to common environmental allergens in plasma samples from recruits in the PROBAT trial for the presence of allergen specific IgE antibodies (Batard *et al.* 1993; Batard *et al.* 1993; Possin *et al.* 2010) is currently being analysed for future work.

Gene expression analysis by quantitative reverse transcriptase PCR of neonatal blood is also necessary for future studies into the role of perinatal probiotic supplementation to determine whether probiotic supplementation effects the gene expression levels of gene transcripts such Epsilon (ϵ) germline gene transcripts $I\epsilon^+$, $C\epsilon^+$ mRNA which precedes class switch recombination to IgE via IL-4 and/or IL-13 (Cameron *et al.* 2000).This would have provided further information of the role of probiotics during infancy at the molecular level.

Furthermore since horizontal transfer of bacterial DNA from mother to foetus via the placenta has been reported and *Bifidobacterium* and *Lactobacillus* and their DNA transcripts have been detected in the human placenta irrespective of mode of delivery: (Satokari *et al.* 2009). It would have been interesting to determine whether

there is a difference in consortium mediated mRNA transcripts in the placenta as a result of perinatal probiotic supplementation.

Additionally the effects of perinatal probiotic supplementation on the immunology of breast milk might also provide some insightful information on the immunomodulatory effects of probiotics in infancy. Since we have archived placental biopsies and milk samples from recruits on the PROBAT trial, such studies can still be initiated.

8.8 Final Conclusion

Th1 responses antagonise the differentiative signature imposed by the upregulation of Th2 responses that support the development of allergy accelerating cytokines such as IL-13. Equally subsequent differentiation of the allergen receptive granulocytes such as the eosinophils and basophils also involved in orchestrating the allergic response is also a consequence. In conclusion the immunomodulatory effect of perinatal administration of a multi-strain probiotic during infancy is marked by a significant reduction in eosinophil counts at birth in the probiotic group, along with a marked reduction in atopic sensitisation and skin prick positivity to common food allergens. Interestingly whole blood cultures from the PROBAT trial recruits stimulated with LPS and IFN-γ induced significantly increased IL-12p70 Th1 immune responses in the probiotic group at 6 months of age. This observation further supports the notion that perinatal probiotic supplementation enhances innate immune function and promotes induction of low grade Th1 mediated inflammation postulated to contribute to the attenuation of the expression of the atopic phenotype in later life. These observations are also indirectly linked to the capacity of the study consortium to down regulates *in vitro* PHA and SEB induced IL-13 (a key allergy orchestrating cytokine) while potentiating IFN-γ (a key Th1 driving cytokine) responses from both PBMCs and CBMCs.

References

Aagaard-Tillery K.M, Silver R, *et al.* (2006). Immunology of normal pregnancy. Seminars in Fetal and Neonatal Medicine 11: 279-295.

Aarvak, T, M. Chabaud, *et al.* (1999). IL-17 is produced by some proinflammatory Th1/Th0 cells but not by Th2 cells. Journal of Immunology 162(3): 1246-1251.

Abrahamsson, T. R, T. Jakobsson, *et al.* (2007). Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. Journal of Allergy and Clinical Immunology 119(5): 1174-1180.

Adkins, B, C. Leclerc, *et al.* (2004). Neonatal adaptive immunity comes of age. Nature Reviews Immunology 4(7): 553-564.

Agace, W. W. (2006). Tissue-tropic effector T cells: generation and targeting opportunities. Nature Reviews Immunology 6(9): 682-692.

Akbar, A. N, L. Terry, *et al.* (1988). Loss of Cd45r and Gain of Uchl1 Reactivity Is a Feature of Primed T-Cells. Journal of Immunology 140(7): 2171-2178.

Akdis, M, J. Verhagen, *et al.* (2004). Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. Journal of Experimental Medicine 199(11): 1567-1575.

Allen, S. J, S. Jordan, *et al.* (2010). Dietary Supplementation with Lactobacilli and Bifidobacteria Is Well Tolerated and Not Associated with Adverse Events during Late Pregnancy and Early Infancy. Journal of Nutrition 140(3): 483-488.

Alm, J. S, J. Swartz, *et al.* (2002[a]). An anthroposophic lifestyle and intestinal microflora in infancy. Journal of Pediatric Allergy and Immunology13(6): 402-411.

Alm, J. S, J. Swartz, *et al.* (1999[b]). Atopy in children of families with an anthroposophic lifestyle. Lancet 353(9163): 1485-1488.

Almqvist, C, B. Wettermark, *et al.* (2012). Antibiotics and asthma medication in a large register-based cohort study - confounding, cause and effect. Clinical and Experimental Allergy 42(1): 104-111.

Anderson, D. J, J. A. Hill, *et al.* (1991). Adverse-Effects of Immune Cell Products in Pregnancy. Molecular and Cellular Immunobiology of the Maternal Fetal Interface: 207-218

Arshad SH, Tariq SM *et al.* (2001). Sensitization to common allergens and its association with allergic disorders at age 4 years: A whole population birth cohort study. Pediatrics 2001;1-8

Asadullah, K, W. Sterry, *et al.* (2003). IL-10 therapy--review of a new approach. Pharmacology Review 55(2): 241-269.

Ashkar, A. A, J. P. Di Santo, *et al.* (2000). Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. Journal of Experimental Medicine 192(2): 259-269.

Bach, J. F. (2002). Mechanisms of disease: The effect of infections on susceptibility to autoimmune and allergic diseases. New England Journal of Medicine 347(12): 911-920.

Backhed, F, H. Ding, *et al.* (2004). The gut microbiota as an environmental factor that regulates fat storage. Proceedings of the National Academy of Sciences of the United States of America 101(44): 15718-15723.

Baraldo, S, K. Lokar Oliani, *et al.* (2007). The Role of Lymphocytes in the Pathogenesis of Asthma and COPD. Current Medicinal Chemistry 14(21): 2250 - 2256.

Barouei, J, M. C. Adams, *et al.* (2009). Prophylactic role of maternal administration of probiotics in the prevention of irritable bowel syndrome. Medical Hypotheses 73(5): 764-767

Barron, J. (1999). The Barron Report: The Probiotic Miracle. Basline of health foundation Volume 7(3).

Barton, L. L. (1984). Infectious-Diseases of the Fetus and Newborn-Infant - Remington *et al.* Journal of the American Medical Association 251(9): 1219-1220.

Basehore, M. J, T. D. Howard, *et al.* (2004). A comprehensive evaluation of IL4 variants in ethnically diverse populations: association of total serum IgE levels and asthma in white subjects. Journal of Allergy and Clinical Immunology 114(1): 80-87.

Batard, T, B. Basuyaux, *et al.* (1993[a]). Isotypic analysis of grass pollen-specific immunoglobulins in human plasma. 1. Specialization of certain classes and subclasses in the immune response. International Archives of Allergy and Immunology 100(1): 68-78.

Batard, T, B. Basuyaux, *et al.* (1993[b]). Isotypic analysis of grass-pollen-specific immunoglobulins in human plasma. 2. Quantification of the IgE, IgM, IgA class and the IgG subclass antibodies. International Archives of Allergy and Immunology 102(3): 279-287.

Beasley, R, U. Keil, *et al.* (1998). Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. Lancet 351(9111): 1225-1232.

Belderbos, M, O. Levy, *et al.* (2009). Neonatal innate immunity in allergy development. Current Opinion Pediatrics 21(6): 762-769.

Berkowitz, R. S, J. A. Hill, *et al.* (1988). Effects of products of activated leukocytes (lymphokines and monokines) on the growth of malignant trophoblast cells *in vitro*. American Journal of Obstetrics and Gynecology 158(1): 199-203.

Berlin, C, R. F. Bargatze, *et al.* (1995). alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. Cell 80(3): 413-422.

Biasucci, G, B. Benenati, *et al.* (2008). Cesarean delivery may affect the early biodiversity of intestinal bacteria. Journal of Nutrition 138(9): 1796s-1800s.

Bieneman AP, Chichester KL, *et al.* (2005). Toll-like receptor 2 ligands activate human basophils for both IgE-dependent and IgE-independent secretion. Journal of Allergy and Clinical Immunology 115(2): 295-301.

Bieber T (2010). Atopic dermatitis. Annals of Dermatology 22: 125–137.

Bjorksten B. (2004[a]). Effects of intestinal microflora and the environment on the development of asthma and allergy. Springer Seminars in Immunopathology 25(3-4): 257-270.

Bjorksten B. (2005[b]). Genetic and environmental risk factors for the development of food allergy. Current Opinion in Allergy and Clinical Immunology 5(3): 249-253.

Bjorksten, B, P. Naaber, *et al.* (1999). The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clinical and Experimental Allergy 29(3): 342-346.

Bjorksten, B, E. Sepp, *et al.* (2001). Allergy development and the intestinal microflora during the first year of life. Journal of Allergy and Clinical Immunology 108(4): 516-520.

Borchers, A. T, C. Selmi, *et al.* (2009). Probiotics and immunity. Journal of Gastroenterology 44(1): 26-46.

Borish, L, A. Aarons, *et al.* (1996). IL-10 regulation in normal subjects and patients with asthma. Journal of Allergy and Clinical Immunology 97(6): 1288-1296.

Borish, L, J. J. Mascali, *et al.* (1992). Detection of Alveolar Macrophage-Derived Il-1-Beta in Asthma - Inhibition with Corticosteroids. Journal of Immunology 149(9): 3078-3082.

Borres, M. P. and B. Bjorksten (2004). Peripheral blood eosinophils and IL-4 in infancy in relation to the appearance of allergic disease during the first 6 years of life.Journal of Pediatric Allergy and Immunology 15(3): 216-20.

Borres, M. P, H. Odelram, *et al.* (1995). Peripheral-Blood Eosinophilia in Infants at 3 Months of Age Is Associated with Subsequent Development of Atopic Disease in Early-Childhood. Journal of Allergy and Clinical Immunology 95(3): 694-698.

Bottcher, M. F, T. R. Abrahamsson, *et al.* (2008). Low breast milk TGF-beta2 is induced by *Lactobacillus reuteri* supplementation and associates with reduced risk of sensitization during infancy. Journal of Pediatric Allergy and Immunology19(6): 497-504.

Bottcher, M. F, M. C. Jenmalm, *et al.* (2006). Cytokine responses to allergens during the first 2 years of life in Estonian and Swedish children. Clinical and Experimental Allergy 36(5): 619-628.

Bottcher, M. F, E. K. Nordin, *et al.* (2000). Microflora-associated characteristics in faeces from allergic and nonallergic infants. Clinical and Experimental Allergy 30(11): 1590-1596.

Bottini, N, P. Borgiani, *et al.* (2002). IL-4 receptor alpha chain genetic polymorphism and total IgE levels in the English population: two-locus haplotypes are more informative than individual SNPs. Clinical Genetics 61(4): 288-292.

Bousquet, J, W. Jacot, *et al.* (2004). Epigenetic inheritance of fetal genes in allergic asthma. Allergy 59(2): 138-147.

Boyle, R. J, L. J. Mah, *et al.* (2008). Effects of Lactobacillus GG treatment during pregnancy on the development of fetal antigen-specific immune responses. Clinical and Experimental Allergy 38(12): 1882-1990.

Braat, H, J. van den Brande, *et al.* (2004). *Lactobacillus rhamnosus* induces peripheral hyporesponsiveness in stimulated $CD4^+$ T cells via modulation of dendritic cell function. American Journal of Clinical Nutrition 80(6): 1618-1625.

Brandtzaeg, P. (2002[a]). Current understanding of gastrointestinal immunoregulation and its relation to food allergy. Genetically Engineered Foods Assessing Potential Allergenicity 964: 13-45.

Brandtzaeg, P. (2009[b]). 'ABC' of mucosal immunology. Nestle Nutrition Workshop Series: Pediatr Program 64: 23-38; discussion 38-43, 251-257.

Braun-Fahrlander, C, J. Riedler, *et al.* (2002). Environmental exposure to endotoxin and its relation to asthma in school-age children. New England Journal of Medicine 347(12): 869-877.

Briskin, M, D. Winsor-Hines, *et al.* (1997). Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue. American Journal of Pathology 151(1): 97-110.

Bromley, S. K, T. R. Mempel, *et al.* (2008). Orchestrating the orchestrators: chemokines in control of T cell traffic. Nature Immunology 9(9): 970-980.

Brouwer, M. L, S. A. A. Wolt-Plompen, *et al.* (2006). No effects of probiotics on atopic dermatitis in infancy: a randomized placebo-controlled trial. Clinical and Experimental Allergy 36(7): 899-906.

Buck, R. H, C. T. Cordle, *et al.* (2002). Longitudinal study of intracellular T cell cytokine production in infants compared to adults. Journal of Clinical Experimental Immunology 128(3): 490-497.

Bullen, C. L, P. V. Tearle, *et al.* (1976). Bifidobacteria in the intestinal tract of infants: an in-vivo study. Journal of Medical Microbiology 9(3): 325-33.

Bullens, D. M. A, E. Truyen, *et al.* (2006). IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? Respiratory Research 7

Bulmer, J. N, L. Morrison, *et al.* (1991). Granulated Lymphocytes in Human Endometrium - Histochemical and Immunohistochemical Studies. Human Reproduction 6(6): 791-798.

Cahill, R. N, D. C. Poskitt, *et al.* (1977). Two distinct pools of recirculating T lymphocytes: migratory characteristics of nodal and intestinal T lymphocytes. Journal of Experimental Medicine 145(2): 420-428.

Calder, P. C. (2007). Immunological parameters: What do they mean? Journal of Nutrition 137(3): 773s-780s.

Cameron, L, Q. Hamid, *et al.* (2000). Local synthesis of epsilon germline gene transcripts, IL-4, and IL-13 in allergic nasal mucosa after ex vivo allergen exposure. Journal of Allergy and Clinical Immunology 106(1): 46-52.

Campbell, D. E, A. S. Fryga, *et al.* (1999). Intracellular interferon-gamma (IFN- gamma) production in normal children and children with atopic dermatitis. Clinical and Experimental Immunology 115(3): 377-382.

Campbell, D. E, D. J. Hill, *et al.* (1998). Enhanced IL-4 but normal interferon-gamma production in children with isolated IgE mediated food hypersensitivity.Journal of Pediatric Allergy and Immunology 9(2): 68-72.

Campbell, D. E. and A. S. Kemp (1997). Proliferation and production IFN-γ and IL-4 in response to Staphylococcus aureus and staphylococcal superantigen in childhood atopic dermatitis. Journal of Clinical Experimental Immunology 107(2): 392-397.

Campbell, J. J, C. E. Brightling, *et al.* (2001). Expression of chemokine receptors by lung T cells from normal and asthmatic subjects. Journal of Immunology 166(4): 2842-2848.

Carr, R. (2000). Neutrophil production and function in newborn infants. British Journal of Haematology 110(1): 18-28.

Cebra, J. J. (1999). Influences of microbiota on intestinal immune system development. American Journal of Clinical Nutrition 69(5): 1046S-1051S.

Celedon, J. C, A. A. Litonjua, *et al.* (2002). Lack of association between antibiotic use in the first year of life and asthma, allergic rhinitis, or eczema at age 5 years. American Journal of Respiratory and Critical Care Medicine 166(1): 72-75.

Chakir, J, J. Shannon, *et al.* (2003). Airway remodeling-associated mediators in moderate to severe asthma: Effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. Journal of Allergy and Clinical Immunology 111(6): 1293-1298.

Chaouat, G, N. Ledee-Bataille, *et al.* (2004). Reproductive immunology 2003: reassessing the Th1/Th2 paradigm? Immunology Letters 92(3): 207-214.

Chaouat, G, E. Menu, *et al.* (1990). Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy. Journal of Reproductiion and Fertility 89(2): 447-58.

Chattopadhyay, S, E. Tracy, *et al.* (2007). IL-31 and oncostatin-M mediate distinct signaling reactions and response patterns in lung epithelial cells. Journal of Biological Chemistry 282(5): 3014-26.

Chelvarajan, R. L, S. M. Collins, *et al.* (2004). Defective macrophage function in neonates and its impact on unresponsiveness of neonates to polysaccharide antigens. Journal of Leukocyte Biology 75(6): 982-994.

Chen, T, P. Isomaki, *et al.* (1999). Human cytokine responses induced by gram-positive cell walls of normal intestinal microbiota. Journal of Clinical Experimental Immunology 118(2): 261-267.

Chipeta, J, Y. Komada, *et al.* (1998). $CD4^+$ and $CD8^+$ cell cytokine profiles in neonates, older children, and adults: increasing T helper type 1 and T cytotoxic type 1 cell populations with age. Cellular Immunology 183(2): 149-56.

Chirico, G. (2005). Development of the Immune System in Neonates. Journal of the Arab Neonatology forum 2: 5-11.

Christensen, H. R, H. Frokiaer, *et al.* (2002). Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. Journal of Immunology 168(1): 171-178.

Christensen, R. D, E. Henry, *et al.* (2009). The CBC: Reference Ranges for Neonates. Seminars in Perinatology 33(1): 3-11.

Chung, H. and D. L. Kasper (2010) Microbiota-stimulated immune mechanisms to maintain gut homeostasis. Current Opinion in Immunology 22(4): 455-60.

Chung, Y, X. Yang, *et al.* (2006). Expression and regulation of IL-22 in the IL-17-producing $CD4^+$ T lymphocytes. Cell Research 16(11): 902-907.

Clark, R. A, B. Chong, *et al.* (2006). The vast majority of CLA(+) T cells are resident in normal skin. Journal of Immunology 176(7): 4431-4439.

Clement, L. T, P. E. Vink, *et al.* (1990). Novel immunoregulatory functions of phenotypically distinct subpopulations of $CD4^+$ cells in the human neonate. Journal of Immunology 145(1): 102-108.

Clement, L. T, N. Yamashita, *et al.* (1988). The functionally distinct subpopulations of human $CD4^+$ helper/inducer T lymphocytes defined by anti-CD45R antibodies derive sequentially from a differentiation pathway that is regulated by activation-dependent post-thymic differentiation. Journal of Immunology 141(5): 1464-70.

Clerici, M, L. DePalma, *et al.* (1993). Analysis of T helper and antigen-presenting cell functions in cord blood and peripheral blood leukocytes from healthy children of different ages. Journal of Clinical Investigation 91(6): 2829-36.

Colonna, M, G. Trinchieri, *et al.* (2004). Plasmacytoid dendritic cells in immunity. Nature Immunology 5(12): 1219-1226.

Comans-Bitter, W. M, R. de Groot, *et al.* (1997). Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. Journal of Pediatrics 130(3): 388-393.

Conrad, M. L, R. Ferstl, *et al.* (2009). Maternal TLR signaling is required for prenatal asthma protection by the nonpathogenic microbe Acinetobacter lwoffii F78. Journal of Experimental Medicine 206(13): 2869-2877.

Constant, S. L, J. L. Brogdon, *et al.* (2002). Resident lung antigen-presenting cells have the capacity to promote Th2 T cell differentiation in situ. Journal of Clinical Investigation 110(10): 1441-1448.

Cookson, W. (2004). The immunogenetics of asthma and eczema: a new focus on the epithelium. Nature Reviews Immunology 4(12): 978-88.

Cooperstock, M. *et al.* (1983). Intestinal flora of infants. In: Hentges DJ, ed Human intestinal microflora in health and disease. New York: Academic Press : 79-99.

Crane, J. (2002). Pro and anti: the biotics of allergic disease. Thorax 57 Supplement 2: II40-II46.

Cross, M. L, L. M. Stevenson, *et al.* (2001). Anti-allergy properties of fermented foods: an important immunoregulatory mechanism of lactic acid bacteria? International Immunopharmacology 1(5): 891-901.

Croy, B. A, S. Chantakru, *et al.* (2002). Decidual natural killer cells: key regulators of placental development (a review). Journal of Reproductive Immunology 57(1-2): 151-168.

Curti, A, S. Trabanelli, *et al.* (2009). The role of indoleamine 2,3-dioxygenase in the induction of immune tolerance: focus on hematology. Blood 113(11): 2394-2401.

Cuss, A. K, D. T. Avery, *et al.* (2006). Expansion of functionally immature transitional B cells is associated with human-immunodeficient states characterized by impaired humoral immunity. Journal of Immunology 176(3): 1506-1516.

de Roock, S, M. van Elk, *et al.* (2010). Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans. Clinical and Experimental Allergy 40(1): 103-110.

de Vries, E, S. de Bruin-Versteeg, *et al.* (2000). Neonatal blood lymphocyte subpopulations: a different perspective when using absolute counts. Biology of the Neonate 77(4): 230-235.

de Vries, E, S. de Bruin-Versteeg, *et al.* (1998). Longitudinal follow-up of blood lymphocyte subpopulations from birth to 1 year of age. Journal of Pediatrics 133(4): 586-8.

De Wit, D, S. Tonon, *et al.* (2003). Impaired responses to toll-like receptor 4 and toll-like receptor 3 ligands in human cord blood. Journal of Autoimmunity 21(3): 277-281.

Deichmann, K, J. Bardutzky, *et al.* (1997). Common polymorphisms in the coding part of the IL-4-receptor gene. Biochemical and Biophysical Research Communications 231(3): 696-697.

Deichmann, K, A. Heinzmann, *et al.* (1998). Linkage and allelic association of atopy and markers flanking the IL4-receptor gene. Clinical and Experimental Allergy 28(2): 151-155.

Deitch, E. A, D. Z. Xu, *et al.* (1991). Bacterial Translocation from the Gut Impairs Systemic Immunity. Surgery 109(3): 269-276.

Delneste, Y, A. Donnet-Hughes, *et al.* (1998). Functional foods: mechanisms of action on immunocompetent cells. Nutrition Reviews 56(1 Pt 2): S93-8.

Delves P, Martin S, *et al.* (2006). Roitt's Essential Immunology. 11th Ed. Chapter title required

Depaoli, P, S. Battistin, *et al.* (1988). Age-Related-Changes in Human-Lymphocyte Subsets - Progressive Reduction of the Cd4 Cd45r (Suppressor Inducer) Population. Journal of Clinical Immunology and Immunopathology 48(3): 290-296.

Diaz-Ropero, M. P, R. Martin, *et al.* (2007). Two Lactobacillus strains, isolated from breast milk, differently modulate the immune response. Journal of Applied Microbiology 102(2): 337-43.

Dillon, S. R, C. Sprecher, *et al.* (2005). IL 31, a cytokine produced by activated T cells, induces dermatitis in mice (vol 5, pg 752, 2004). Nature Immunology 6(1): 114-114.

Drake, B *et al.* (1990). Murine trophoblast cells are not killed by tumor necrosis factor-alpha. Journal of reproductive Immuology 17(1): 93-99.

Drakes, M, T. Blanchard, *et al.* (2004). Bacterial probiotic modulation of dendritic cells. Infection and Immunity 72(6): 3299-3309.

Drouault-Holowacz, S, B. Foligne, *et al.* (2006). Anti-inflammatory potential of the probiotic dietary supplement Lactibiane Tolerance: *in vitro* and *in vivo* considerations. Clinical Nutr 25(6): 994-1003.

Early, E. and D. J. Reen (1999). Rapid conversion of naive to effector T cell function counteracts diminished primary human newborn T cell responses. Clinical and Experimental Immunology 116(3): 527-533.

Eckburg, P. B, E. M. Bik, *et al.* (2005). Diversity of the human intestinal microbial flora. Science 308(5728): 1635-1638.

Eisenhofer, G, A. Aneman, *et al.* (1997). Substantial production of dopamine in the human gastrointestinal tract. Journal of Clinical Endocrinology & Metabolism 82(11): 3864-3871.

Elazab, N. Mendy *et al.* (2013) Probiotic administration in early life, Atopy, and Asthma: a meta-analysis of clinical trials. Pediatrics 132: 666–676.

Erkeller-Yuksel, F. M, V. Deneys, *et al.* (1992). Age-related changes in human blood lymphocyte subpopulations. Journal of Pediatrics 120(2 Pt 1): 216-222.

Falk, P. G, L. V. Hooper, *et al.* (1998). Creating and maintaining the gastrointestinal ecosystem: What we know and need to know from gnotobiology. Microbiology and Molecular Biology Reviews 62(4): 1157-1170.

Fallon, P. G, H. E. Jolin, *et al.* (2002). IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. Immunity 17(1): 7-17.

Farstad, I. N, T. S. Halstensen, *et al.* (1996). Distribution of beta 7 integrins in human intestinal mucosa and organized gut-associated lymphoid tissue. Immunology 89(2): 227-237.

Felis, G. E. and F. Dellaglio (2007). Taxonomy of Lactobacilli and Bifidobacteria. Current Issues in Intestestinal Microbiology 8(2): 44-61.

Ferrer, J. M, A. Plaza, *et al.* (1992). Differential IL secretion by *in vitro* activated human CD45RA and CD45RO $CD4^+$ T cell subsets. Cellular Immunology141(1): 10-20.

Fink, L. N, L. H. Zeuthen, *et al.* (2007). Distinct gut-derived lactic acid bacteria elicit divergent dendritic cell-mediated NK cell responses. International Immunology 19(12): 1319-1327.

Fiocchi A, Burks W, Bahna SL, et al. Clinical use of probiotics in pediatric allergy (CUPPA): a world allergy organization position paper. World Allergy Organ J2012;5:148–67.

Fitzgerald, J. S, T. G. Poehlmann, *et al.* (2008). Trophoblast invasion: the role of intracellular cytokine signalling via signal transducer and activator of transcription 3 (STAT3). Human Reproductive Update 14(4): 335-344.

Fitzgerald K, O'neil L, *et al.* (2001). The cytokine facts book. 2nd Edition: 1-515.

Flinterman, A. E, E. F. Knol, *et al.* (2007). Probiotics have a different immunomodulatory potential *in vitro* versus ex vivo upon oral administration in children with food allergy. International Archives of Allergy and Immunology 143(3): 237-244.

Foligne, B, S. Nutten, *et al.* (2007). Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World Journal of Gastroenterology 13(2): 236-243.

Flohr C (2011). Recent perspectives on the global epidemiology of childhood eczema. Allergologia et Immunopathologia (Madr) 39:174–182.

Foligne, B, G. Zoumpopoulou, *et al.* (2007). A Key Role of Dendritic Cells in Probiotic Functionality. PLoS One 2(3).

Fontenot, J. D, M. A. Gavin, *et al.* (2003). Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. Nature Immunology 4(4): 330-336.

Fort, M. M, J. Cheung, *et al.* (2001). IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. Immunity 15(6): 985-995.

Fossiez, F, O. Djossou, *et al.* (1996). T cell IL-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. Journal of Experimental Medicine 183(6): 2593-2603.

Foster, P. (2003). IL-13 and allergy. Mod. Asp. Immunobiol. 3(1): 1.

Frenkel, L. and Y. J. Bryson (1987). Ontogeny of Phytohemagglutinin-Induced Gamma Interferon by Leukocytes of Healthy Infants and Children - Evidence for Decreased Production in Infants Younger Than 2 Months of Age. Journal of Pediatrics 111(1): 97-100.

Fukushima, Y, Y. Kawata, *et al.* (1999). Effect of bifidobacteria feeding on fecal flora and production of immunoglobulins in lactating mouse. International Journal of Food Microbiology 46(3): 193-197.

Furrie, E. (2005). Probiotics and allergy. Proceedings of the Nutrition Society 64(4):465-469.

Galli, S. J, S. Nakae, *et al.* (2005). Mast cells in the development of adaptive immune responses. Nature Immunology 6(2): 135-142.

Gasparoni, A, L. Ciardelli, *et al.* (2003). Age-related changes in intracellular TH1/TH2 cytokine production, immunoproliferative T lymphocyte response and natural killer cell activity in newborns, children and adults. Biology of the Neonate 84(4): 297-303.

Gauvreau, G. M, A. K. Ellis, *et al.* (2009). Haemopoietic processes in allergic disease: eosinophil/basophil development. Clinical and Experimental Allergy 39(9): 1297-306.

Gauvreau, G. M, J. M. Lee, *et al.* (2000). Increased numbers of both airway basophils and mast cells in sputum after allergen inhalation challenge of atopic asthmatics. American Journal of Respiratory and Critical Care Medicine 161(5): 1473-1478.

Ghadimi, D, M. de Vrese, *et al.* (2010). Lactic acid bacteria enhance autophagic ability of mononuclear phagocytes by increasing Th1 autophagy-promoting cytokine (IFN-γ) and nitric oxide (NO) levels and reducing Th2 autophagy-restraining cytokines (IL-4 and IL-13) in response to Mycobacterium tuberculosis antigen. International ImmunoPharmacology 10(6): 694-706.

Gibbs, B. F. (2005). Human basophils as effectors and immunomodulators of allergic inflammation and innate immunity. Clinical and Experimental Medicine 5(2): 43-49.

Gill, H. S. (1998). Stimulation of the immune system by lactic cultures. International Dairy Journal 8(5-6): 535-544.

Gill, H. S, K. J. Rutherfurd, *et al.* (2001). Dietary probiotic supplementation enhances natural killer cell activity in the elderly: An investigation of age-related immunological changes. Journal of Clinical Immunology 21(4): 264-271.

Gillingham, L. G. and D. Lescheid (2009). Probiotics and Mucosal Immunity:Strain-specific effects on Th1/Th2 cell modulation. International Journal of Naturopathic Medicine 4(1): 18-22.

Girardi, G, R. Bulla, *et al.* (2006). The complement system in the pathophysiology of pregnancy. Molecular Immunology 43(1-2): 68-77.

Godfrey, W. R, D. J. Spoden, *et al.* (2005). Cord blood $CD4^{+}CD25^{+}$-derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function. Blood 105(2): 750-758.

Gold, M. S. and A. S. Kemp (2005). Atopic disease in childhood. Med Journal of Australia 182(6): 298-304.

Gorfu, G, J. Rivera-Nieves, *et al.* (2009). Role of beta7 integrins in intestinal lymphocyte homing and retention. Current Molecular Medicine 9(7): 836-50.

Goriely, S, C. Van Lint, *et al.* (2004). A defect in nucleosome remodeling prevents IL-12(p35) gene transcription in neonatal dendritic cells. Journal of Experimental Medicine 199(7): 1011-1016.

Gould, H. J, B. J. Sutton, *et al.* (2003). The biology of IgE and the basis of allergic disease. Annual Review of Immunology 21: 579-628.

Grangette, C, E. Macho-Fernandez, *et al.* (2012). Anti-inflammatory capacity of lactobacilli peptidoglycan: mucosal and systemic routes of administration promote similar effects - The Authors' reply. Gut 61(5): 784-784.

Grewe, M, C. A. Bruijnzeel-Koomen, *et al.* (1998). A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. Immunology Today 19(8): 359-61.

Gronlund, M. M, H. Arvilommi, *et al.* (2000). Importance of intestinal colonisation in the maturation of humoral immunity in early infancy: a prospective follow up study of healthy infants aged 0-6 months. Archives of Disease in Childhood Fetal Neonatal Ed 83(3): F186-192.

Guarner, F. and A. Khan (2008). Probiotics and Prebiotics. World Gastroenterology Organisation Practice Guideline 1-22.

Guarner, F. and J. R. Malagelada (2003[a]). Bacterial flora of the digestive tract. Gastroenterology and Hepatology 26 Suppl 1: 1-5.

Guarner, F. and J. R. Malagelada (2003[b]). Gut flora in health and disease. Lancet 361(9356): 512-519.

Guarner, F. and G. J. Schaafsma (1998). Probiotics. International Journal of Food Microbiology 39(3): 237-238.

Gueimonde, M, K. Laitinen, *et al.* (2007). Breast milk: a source of bifidobacteria for infant gut development and maturation? Neonatology 92(1): 64-66.

Gustafsson, C, J. Mjosberg, *et al.* (2008). Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype. PLoS One 3(4): e2078.

Hadis, U, B. Wahl, *et al.* (2011). Intestinal tolerance requires gut homing and expansion of $FoxP3^+$ regulatory T cells in the lamina propria. Immunity 34(2): 237-246.

Hagendorens, M. M, D. G. Ebo, *et al.* (2003). Differences in circulating dendritic cell subtypes in cord blood and peripheral blood of healthy and allergic children. Clinical and Experimental Allergy 33(5): 633-639.

Hakonarson, H, D. J. Herrick, *et al.* (1997). Autocrine role of IL-1 beta in altered responsiveness of atopic asthmatic sensitized airway smooth muscle. Journal of Clinical Investigation 99(1): 117-124.

Hall, M. A, C. B. Cole, *et al.* (1990). Factors influencing the presence of faecal lactobacilli in early infancy. Archives of Disease in Childhood 65(2): 185-8.

Haller D (2000). Activation of human peripheral blood mononuclear cells by non pathogenic bacteria *in vitro*: evidence of NK cells as primary targets. 68: 752-759.

Haller, D, S. Blum, *et al.* (2000). Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria *in vitro*: Evidence of NK cells as primary targets. Infection and Immunity 68(2): 752-759.

Harrington, L. E, R. D. Hatton, *et al.* (2005). IL 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nature Immunology 6(11): 1123-1132.

Harris, D. T, M. J. Schumacher, *et al.* (1992). Phenotypic and Functional Immaturity of Human Umbilical-Cord Blood Lymphocytes-T. Proceedings of the National Academy of Sciences of the United States of America 89(21): 10006-10010.

Hart, A. L, K. Lammers, *et al.* (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut 53(11): 1602-1609.

Hartel, C, N. Adam, *et al.* (2005). Cytokine responses correlate differentially with age in infancy and early childhood. Clinical and Experimental Immunology 142(3): 446-453.

Hashimoto, T, K. Akiyama, *et al.* (2005). Comparison of IL-17 production by helper T cells among atopic and nonatopic asthmatics and control subjects. International Archives of Allergy and Immunology 137: 51-54.

Hathaway, L. J. and J. P. Kraehenbuhl (2000). The role of M cells in mucosal immunity. Cellular and Molecular Life Sciences 57(2): 323-332.

Hawrylowicz, C. M. and A. O'Garra (2005). Potential role of IL-10-secreting regulatory T cells in allergy and asthma. Nature Reviews Immunology 5(4): 271-283.

Hayward, A. R. and A. R. Lawton (1977). Induction of plasma cell differentiation of human fetal lymphocytes: evidence for functional immaturity of T and B cells. Journal of Immunology 119(4): 1213-1217.

Heaton, T, J. Rowe, *et al.* (2005). An immunoepidemiological approach to asthma: identification of *in vitro* T-cell response patterns associated with different wheezing phenotypes in children. Lancet 365(9454): 142-149.

Hehlgans, T. and K. Pfeffer (2005). The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology 115(1): 1-20.

Hellings, P. W, A. Kasran, *et al.* (2003). IL-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. American Journal of Respiratory Cell and Molecular Biology 28(1): 42-50.

Helwig, U, K. M. Lammers, *et al.* (2006). Lactobacilli, bifidobacteria and E. coli nissle induce pro- and anti-inflammatory cytokines in peripheral blood mononuclear cells. World Journal of Gastroenterology 12(37): 5978-5986.

Henneke, P. and R. Berner (2006). Interaction of neonatal phagocytes with group B streptococcus: Recognition and response. Infection and Immunity 74(6): 3085-3095.

Herberth, G, J. Heinrich, *et al.* (2010). Reduced IFN-gamma- and enhanced IL-4-producing $CD4^+$ cord blood T cells are associated with a higher risk for atopic dermatitis during the first 2 yr of life. Pediatric Allergy and Immunology 21(1): 5-13.

Hermiston ML, Xu Z, *et al.* (2003). CD45: A critical regulator of signalling thresholds in immune cells. Annual Reviews Immunology 21: 107-371.

Hershey, G. K, M. F. Friedrich, *et al.* (1997). The association of atopy with a gain-of-function mutation in the alpha subunit of the IL-4 receptor. New England Journal of Medicine 337(24): 1720-1725.

Hessle, C, L. A. Hanson, *et al.* (1999). Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Journal of Clinical Experimental Immunology 116(2): 276-282.

Hoeger, P. H, W. Lenz, *et al.* (1992). Staphylococcal skin colonization in children with atopic dermatitis: prevalence, persistence, and transmission of toxigenic and nontoxigenic strains. Journal of Infectious Disease 165(6): 1064-1068.

Hofer, M. F, R. J. Harbeck, *et al.* (1999). Staphylococcal toxins augment specific IgE responses by atopic patients exposed to allergen. Journal of Investigative Dermatology 112(2): 171-176.

Holgate, S. T. (2000). Epithelial damage and response. Clinical and Experimental Allergy 30: 37-41.

Holt, P. G, J. B. Clough, *et al.* (1992). Genetic Risk for Atopy Is Associated with Delayed Postnatal Maturation of T-Cell Competence. Clinical and Experimental Allergy 22(12): 1093-1099.

Holt, P. G, C. Macaubas, *et al.* (1999). Microbial stimulation as an aetiologic factor in atopic disease. Allergy 54: 12-16.

Holt, P. G, J. W. Upham, *et al.* (2005). Contemporaneous maturation of immunologic and respiratory functions during early childhood: Implications for development of asthma prevention strategies. Journal of Allergy and Clinical Immunology 116(1): 16-24.

Hooper, L. V. and A. J. Macpherson (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. Nature Reviews Immunology 10(3): 159-169.

Hoshino, H, M. Laan, *et al.* (2000). Increased elastase and myeloperoxidase activity associated with neutrophil recruitment by IL-17 in airways *in vivo*. Journal of Allergy and Clinical Immunology 105(1): 143-149.

Hunt, J. S, J. L. Fishback, *et al.* (1990). Identification of class I MHC mRNA in human first trimester trophoblast cells by in situ hybridization. Journal of Immunology 144(11): 4420-4425.

Hunt, J. S, D. L. Lessin, *et al.* (1989). Ontogeny and Distribution of Cells Expressing Hla-B Locus-Specific Determinants in the Placenta and Extraplacental Membranes. Journal of Reproductive Immunology 15(1): 21-30.

Huurre, A, K. Laitinen, *et al.* (2008). Impact of maternal atopy and probiotic supplementation during pregnancy on infant sensitization: a double-blind placebo-controlled study. Clinical and Experimental Allergy 38(8): 1342-1348.

Huynh, H. Q, J. deBruyn, *et al.* (2009). Probiotic preparation VSL#3 induces remission in children with mild to moderate acute ulcerative colitis: a pilot study. Inflammatory Bowel Disease 15(5): 760-768.

Hytonen, A. M, O. Lowhagen, *et al.* (2004). Haplotypes of the IL-4 receptor alpha chain gene associate with susceptibility to and severity of atopic asthma. Clinical and Experimental Allergy 34(10): 1570-1575.

Ichikawa, S, M. Miyake, *et al.* (2009). Orally Administered *Lactobacillus paracasei* KW3110 Induces *in vivo* IL-12 Production. Bioscience Biotechnology and Biochemistry 73(7): 1561-1565.

Iliopoulos, O, F. M. Baroody, *et al.* (1992). Histamine-Containing Cells Obtained from the Nose Hours after Antigen Challenge Have Functional and Phenotypic Characteristics of Basophils. Journal of Immunology 148(7): 2223-2228.

Illi S, von Mutius E, *et al.* (2001[a]). Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. BMJ 322(7283): 390-395.

Illi S, von Mutius E, *et al.* (2001[b]). The pattern of atopic sensitization is associated with the development of asthma in childhood. Journal of Allergy and Clinical Immunology 108(5): 709-714.

Illi S, von Mutius E *et al.* (2006[c]) Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. Lancet 2006; 368:763–770.

Ishikawa H, Tanaka K. *et al.* (2008). Effect of intestinal microbiota on the induction of regulatory $CD25^+$ $CD4^+$ T cells. Clinical and Experimental Immunology 153(1): 127-135.

Isidoro-Garcia M, Davila I, *et al.* (2005). IL-4 (IL-4) and IL-4 receptor (IL-4RA) polymorphisms in asthma: a case control study. Clinical Molecular Allergy 3: 15.

Islam SA and Luster AD (2012). T cell homing to epithelial barriers in allergic disease. Nature Medicine 18(5): 705-715.

Isolauri E (2004). The role of probiotics in paediatrics. Current paediatrics 14(2): 104-109.

Isolauri, E, T. Arvola, *et al.* (2000). Probiotics in the management of atopic eczema. Clinical and Experimental Allergy 30(11): 1604-10.

Isolauri, E, J. Joensuu, *et al.* (1995). Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. Vaccine 13(3): 310-312.

Isolauri, E, M. Kalliomaki, *et al.* (2009). Obesity - extending the hygiene hypothesis. Nestle Nutrition Workshop Series: Pediatric Program 64: 75-85; discussion 85-89, 251-257.

Isolauri, E, Y. Sutas, *et al.* (2001). Probiotics: effects on immunity. American Journal of Clinical Nutrition 73(2 Supplement): 444S-450S.

Iwasaki, A. and B. L. Kelsall (1999). Freshly isolated Peyer's patch, but not spleen, dendritic cells produce IL 10 and induce the differentiation of T helper type 2 cells. Journal of Experimental Medicine 190(2): 229-239.

Janeway, C. (2003). Chapter 5: Antigen presentation to T lymphocytes. Immunobiology: The immune system in health and disease: 193.

Janeway, C. A, Jr. (1992). The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. Annual Reviews Immunology 10: 645-74.

Jarvis, D. and P. Burney (1998). ABC of allergies. The epidemiology of allergic disease. British Medical Journal 316(7131): 607-610.

Joetham, A, K. Takada, *et al.* (2007). Naturally occurring lung $CD4^{+}CD25^{+}$ T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta. Journal of Immunology 178(8): 14331442.

Joffe, M. I, M. Kew, *et al.* (1983). Lymphocyte Subtypes in Patients with Atopic Eczema, Protein Calorie Malnutrition, SLE and Liver-Disease. Journal of Clinical & Laboratory Immunology 10(2): 97-101.

Johansson, S. G, T. Bieber, *et al.* (2004). Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. Journal of Allergy and Clinical Immunology 113(5): 832-836.

Johansson, S. G. and J. Lundahl (2001). Asthma, atopy, and IgE: what is the link? Current Allergy and Asthma Reports 1(2): 89-90.

Jones, C. A, J. J. FinlayJones, *et al.* (1997). Type-1 and type-2 cytokines in human late-gestation decidual tissue. Biology of Reproduction 57(2): 303-311.

Jordan, S, M. Storey, *et al.* (2008). Antibiotics and allergic disorders in childhood. The Open Nursing Journal 2: 48-57.

Jordan S, Watkins A, Storey M, et al. Volunteer bias in recruitment, retention, and blood sample donation in a randomised controlled trial involving mothers and their children at six months and two years: a longitudinal analysis. PLoS ONE 8: e67912.

Kabesch, M. and R. P. Lauener (2004). Why Old McDonald had a farm but no allergies: genes, environments, and the hygiene hypothesis. Journal of Leukocyte Biology 75(3): 383-387.

Kaila, M, E. Isolauri, *et al.* (1995). Viable versus inactivated Lactobacillus strain GG in acute rotavirus diarrhoea. Archives of Disease in Childhood 72(1): 51-53.

Kaila, M, E. Isolauri, *et al.* (1992). Enhancement of the Circulating Antibody Secreting Cell Response in Human Diarrhea by a Human Lactobacillus Strain. Pediatric Research 32(2): 141-144.

Kalinski, P, C. M. U. Hilkens, *et al.* (1999). T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunology Today 20(12): 561-567.

Kalliomäki M, Kirjavainen P, *et al.* (2001). Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. Journal of Allergy and Clinical Immunology 107: 129-134.

Kalliomaki, M, S. Salminen, *et al.* (2001[a]). Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet 357(9262): 1076-1079.

Kalliomaki, M, S. Salminen, *et al.* (2003[b]). Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. Lancet 361(9372): 1869-1871.

Kalliomaki, M, S. Salminen, *et al.* (2007[c]). Probiotics during the first 7 years of life: a cumulative risk reduction of eczema in a randomized, placebo-controlled trial. Journal of Allergy and Clinical Immunology 119(4): 1019-21.

Kalliomaki, M. A. and E. Isolauri (2004). Probiotics and down-regulation of the allergic response. Immunology and Allergy Clinics of North America 24(4): 739.

Karlsson, H, C. Hessle, *et al.* (2002). Innate immune responses of human neonatal cells to bacteria from the normal gastrointestinal flora. Infection and Immunity 70(12): 6688-6696.

Kato, I, K. Tanaka, *et al.* (1999). Lactic acid bacterium potently induces the production of IL-12 and interferon-gamma by mouse splenocytes. International Journal of Immunology and Pharmacology 21(2): 121-131.

Kauppi, P, K. Lindblad-Toh, *et al.* (2001). Second-generation association study of the 5q31 cytokine gene cluster and the IL-4 receptor in asthma. Genomics 77(1-2): 35-42.

Kaur, B, H. R. Anderson, *et al.* (1998). Prevalence of asthma symptoms, diagnosis, and treatment in 12-14 year old children across Great Britain (international study of asthma and allergies in childhood, ISAAC UK). British Medical Journal 316(7125): 118-124.

Kekkonen, R. A, E. Kajasto, *et al.* (2008). Probiotic Leuconostoc mesenteroides ssp. cremoris and Streptococcus thermophilus induce IL-12 and IFN-gamma production. World Journal of Gastroenterology 14(8): 1192-1203.

Kelsall, B. L. and F. Leon (2005). Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease. Immunological Reviews 206: 132-148.

Kenzel, S. and P. Henneke (2006). The innate immune system and its relevance to neonatal sepsis. Current Opinion in Infectious Disease 19(3): 264-270.

Ker J, Hartert TV. The atopic march: what's the evidence? Ann Allergy Asthma Immunol 2009;103:282–9.

Kim, J. Y, J. H. Kwon, *et al.* (2009). Effect of probiotic mix *(Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus)* in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial.Journal of Pediatric Allergy and Immunology2 1(2 Pt 2): e386-393.

King, A. and Y. W. Loke (1991). On the Nature and Function of Human Uterine Granular Lymphocytes. Immunology Today 12(12): 432-435.

Kirjavainen, P. V, E. Apostolou, *et al.* (1999). New aspects of probiotics:a novel approach in the management of food allergy. Allergy 54(9): 909-915.

Koch, C. and J. Platt (2007). T cell recognition and immunity in the fetus and mother. Cellular Immunology 248(1): 12-17.

Kopp, M. V, M. Goldstein, *et al.* (2008). Lactobacillus GG has *in vitro* effects on enhanced IL-10 and interferon-gamma release of mononuclear cells but no *in vivo* effects in supplemented mothers and their neonates. Clinical and Experimental Allergy 38(4): 602-610.

Kopp, M. V, I. Hennemuth, *et al.* (2008). Randomized, double-blind, placebo-controlled trial of probiotics for primary prevention: No clinical effects of *Lactobacillus GG* supplementation. Pediatrics 121(4): E850-E856.

Koshino, T, Y. Arai, *et al.* (1996). Airway basophil and mast cell density in patients with bronchial asthma: Relationship to bronchial hyperresponsiveness. Journal of Asthma 33(2): 89-95.

Krishnan, L, L. J. Guilbert, *et al.* (1996). T helper 1 response against Leishmania major in pregnant C57BL/6 mice increases implantation failure and fetal resorptions - Correlation

with increased IFN-gamma and TNF and reduced IL-10 production by placental cells. Journal of Immunology 156(2): 653-662.

Krishnan, S, M. Craven, *et al.* (2003). Differences in participation of innate and adaptive immunity to respiratory syncytial virus in adults and neonates. Journal of Infectious Disease 188(3): 433-439.

Kruse, S, T. Japha, *et al.* (1999). The polymorphisms S503P and Q576R in the IL-4 receptor alpha gene are associated with atopy and influence the signal transduction. Immunology 96(3): 365-71.

Kuitunen, M, K. Kukkonen, *et al.* (2009). Probiotics prevent IgE-associated allergy until age 5 years in cesarean-delivered children but not in the total cohort. Journal of Allergy and Clinical Immunology 123(2): 335-41.

Kukkonen, K, E. Savilahti, *et al.* (2007). Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. Journal of Allergy and Clinical Immunology 119(1): 192-198.

Kumar, R. K, C. Herbert, *et al.* (2003). Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma. Journal of Pharmacolog and Experimental Therapeutics 307(1): 349-355.
Kunz B, Oranje AP, Labrèze L, *et al.* (1997) Clinical validation and guidelines for the SCORAD index: consensus report of the European Task Force on Atopic Dermatitis. Dermatology (195):10–19.

Kuriakose, J. S. and R. L. Miller (2010). Environmental epigenetics and allergic diseases: recent advances. Clinical and Experimental Allergy 40(11): 1602-1610.

Kurukulaaratchy, R. J, S. Matthews, *et al.* (2005). Defining childhood atopic phenotypes to investigate the association of atopic sensitization with allergic disease. Allergy 60(10): 1280-1296.

Lamacchia, C, G. Palmer, *et al.* (2010). Enhanced Th1 and Th17 Responses and Arthritis Severity in Mice With a Deficiency of Myeloid Cell-Specific IL-1 Receptor Antagonist. Arthritis and Rheumatism 62(2): 452-462.

Lammers, K. M, P. Brigidi, *et al.* (2003). Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. Fems Immunology and Medical Microbiology 38(2): 165-172.

Lebeer, S, J. Vanderleyden, *et al.* (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nature Reviews Microbiology 8(3): 171-184.

Leber, A, A. Teles, *et al.* (2010). Regulatory T Cells and Their Role in Pregnancy. American Journal of Reproductive Immunology 63(6): 445-459.

Lee, S. G, B. S. Kim, *et al.* (2004). Gene-gene interaction between IL-4 and IL-4 receptor alpha in Korean children with asthma. Clinical and Experimental Allergy 34(8): 1202-1208.

Leigh, R, R. Ellis, *et al.* (2004). Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. American Journal of Respiratory and Critical Care Medicine169(7): 860-7.

Lequin, R. (2005). Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA).Clinical Chemistry 51(12): 2415-2418.

Leung, D. Y, R. Harbeck, *et al.* (1993). Presence of IgE antibodies to staphylococcal exotoxins on the skin of patients with atopic dermatitis. Evidence for a new group of allergens. Journal of Clinical Investigation 92(3): 1374-1380.

Leung DY, Bieber T (2003). Atopic dermatitis. Lancet 361:151–160.

Levi-Schaffer, F, V. Temkin, *et al.* (1998). Mast cells enhance eosinophil survival *in vitro*: Role of TNF-alpha and granulocyte-macrophage colony-stimulating factor. Journal of Immunology 160(11): 5554-5562.

Levy, O. (2007). Innate immunity of the newborn: basic mechanisms and clinical correlates. Nature Reviews Immunology 7(5): 379-390.

Lewis, D. B. (1998). Cellular immunity of the human fetus and neonate. Immunology and Allergy Clinics of North America 18(2): 291-328.

Lewis, D. B, C. C. Yu, *et al.* (1991). Cellular and Molecular Mechanisms for Reduced IL-4 and Interferon-Gamma Production by Neonatal T-Cells. Journal of Clinical Investigation 87(1): 194-202.

Lim, S, E. Crawley, *et al.* (1998). Haplotype associated with low IL-10 production in patients with severe asthma. Lancet 352(9122): 113.

Lima, J. O, L. Zhang, *et al.* (2000). Early expression of epsilon, CD23 (FcepsilonRII), IL-4R-α, and IgE in the human fetus. Journal of Allergy and Clinical Immunology 106(5): 911-917.

Lin, H, T. R. Mosmann, *et al.* (1993). Synthesis of T-Helper 2-Type Cytokines at the Maternal-Fetal Interface. Journal of Immunology 151(9): 4562-4573.

Liu, A. H. and D. Y. M. Leung (2006). Renaissance of the hygiene hypothesis. Journal of Allergy and Clinical Immunology 117(5): 1063-1066.

Liu, W. H, A. L. Putnam, *et al.* (2006). CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ T reg cells. Journal of Experimental Medicine 203(7): 1701-1711.

Liu, Y. J, C. Arpin, *et al.* (1996). Sequential triggering of apoptosis, somatic mutation and isotype switch during germinal center development. Seminars in Immunology 8(3): 169-177.

Loken, M. R, J. M. Brosnan, *et al.* (1990). Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. Cytometry 11(4): 453-459.

Lonnerdal, B. (2010). Novel Insights into Human Lactation as a Driver of Infant Formula Development. Drivers of Innovation in Pediatric Nutrition 66: 19-29

Lund, J. M, M. M. Linehan, *et al.* (2006). Cutting edge: Plasmacytoid dendritic cells provide innate immune protection against mucosal viral infection in situ. Journal of Immunology 177(11): 7510-7514.

Lundequist, B, C. E. Nord, *et al.* (1985). The composition of the faecal microflora in breastfed and bottle fed infants from birth to eight weeks. Acta paediatrica Scandinavica 74(1): 45-51.

Luster, A. D, R. Alon, *et al.* (2005). Immune cell migration in inflammation: present and future therapeutic targets. Nature Immunology 6(12): 1182-1190.

Macaubas, C, P. D. Sly, *et al.* (1999). Regulation of T-helper cell responses to inhalant allergen during early childhood. Clinical and Experimental Allergy 29(9): 1223-1331.

Macfarlane, A. J, O. M. Kon, *et al.* (2000). Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. Journal of Allergy and Clinical Immunology 105(1 Pt 1): 99-107.

Mackie, R. I, A. Sghir, *et al.* (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. American Journal of Clinical Nutrition 69(5): 1035S-1045S.

Macpherson, A. J, M. B. Geuking, *et al.* (2005). Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria. Immunology 115(2): 153-162.

Makhseed, M, R. Raghupathy, *et al.* (2001). Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and with subsequent abortions. Human Reproduction 16(10): 2219-2226.

Mangan, P. R, L. E. Harrington, *et al.* (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. Nature 441(7090): 231-234.

Marchant, A. and M. Goldman (2005). T cell-mediated immune responses in human newborns: ready to learn? Clinical and Experimental Immunology 141(1): 10-18.

Marchini, G, A. Nelson, *et al.* (2005). Erythema toxicum neonatorum is an innate immune response to commensal microbes penetrated into the skin of the newborn infant. Pediatric Research 58(3): 613-616.

Marie JC, R. A. (2006). Cellular mechanism of fatal early onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. Immunity 25(3): 441-454.

Marschan, E. (2007). Immunological effects of probiotic bacteria in prevention and treatment of allergic diseases in Children. Publications of the National Public Health Institute: 1-131.

Marschan, E, M. Kuitunen, *et al.* (2008). Probiotics in infancy induce protective immune profiles that are characteristic for chronic low-grade inflammation. Clinical and Experimental Allergy 38(4): 611-618.

Marsh, D. G, J. D. Neely, *et al.* (1994). Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. Science 264(5162): 1152-1156.

Martin, R, E. Jimenez, *et al.* (2006). *Lactobacillus salivarius* CECT 5713, a potential probiotic strain isolated from infant feces and breast milk of a mother-child pair. International Journal of Food Microbiology 112(1): 35-43.

Martinez, F. D. (1994). Role of Viral-Infections in the Inception of Asthma and Allergies during Childhood - Could They Be Protective. Thorax 49(12): 1189-1191.

Martino, D. J. and S. L. Prescott (2010). Silent mysteries: epigenetic paradigms could hold the key to conquering the epidemic of allergy and immune disease. Allergy 65(1): 7-15.

Masoli, M, D. Fabian, *et al.* (2004). The global burden of asthma: executive summary of the GINA Dissemination Committee Report. Allergy 59(5): 469-478.

Masopust, D, D. Choo, *et al.* (2010). Dynamic T cell migration program provides resident memory within intestinal epithelium. Journal of Experimental Medicine 207(3): 553-564.

Matsuda, H, T. Suda, *et al.* (2002). Alteration of balance between myeloid dendritic cells and plasmacytoid dendritic cells in peripheral blood of patients with asthma. American Journal of Respiratory and Critical Care Medicine 166(8): 1050-1054.

Matsuguchi, T, A. Takagi, *et al.* (2003). Lipoteichoic acids from Lactobacillus strains elicit strong tumor necrosis factor alpha-inducing activitives in macrophages through toll-like receptor 2. Clinical and Diagnostic Laboratory Immunology 10(2): 259-266.

Matsumoto, K, Y. Shimanouchi, *et al.* (2005). Infantile eczema at one month of age is associated with cord blood eosinophilia and subsequent development of atopic dermatitis and wheezing illness until two years of age. International Archives of Allergy and Immunology 137: 69-76.

Mazmanian, S. K, C. H. Liu, *et al.* (2005). An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 122(1): 107-118.

McCracken, V. J. and R. G. Lorenz (2001). The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. Cellular Microbiology 3(1): 1-11.

McDonagh, S, E. Maidji, *et al.* (2004). Viral and bacterial pathogens at the maternal-fetal interface. Journal of Infectious Diseases 190(4): 826-834.

McLoughlin, R. M. and K. H. G. Mills (2011). Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthmaJournal of Allergy and Clinical Immunology 128(1): 253-253.

Metchnikoff, E. (1908). The prolongation of life. Optimistic studies.

Miettinen, M. (1998). Lactobacilli and Streptococci Induce IL-12, IL-12, IL-18, and Gamma Interferon Production in Human Peripheral Blood Mononuclear Cells. Infection and Immunity 66(12): 6058-6062.

Miettinen, M, A. Lehtonen, *et al.* (2000). Lactobacilli and streptococci activate NF-κB and STAT signaling pathways in human macrophages. Journal of Immunology 164(7): 3733-3740.

Miettinen M, (1996). Production of human TNF-α, IL-6 and IL-10 is induced by lactic acid bacteria Infection and Immunology 64 5403-5405.

Miles, E. A, J. A. Warner, *et al.* (1996). Peripheral blood mononuclear cell proliferative responses in the first year of life in babies born to allergic parents. Clinical and Experimental Allergy 26(7): 780-788.

Miller, D. L, T. Hiravonen, *et al.* (1973). Synthesis of IgE by the human conceptus. Journal of Allergy and Clinical Immunology 52(3): 182-188.

Miller, E. M. (2009). Changes in Serum Immunity During Pregnancy. American Journal of Human Biology 21(3): 401-403.

Miller, R. L. and S. M. Ho (2008). Environmental epigenetics and asthma: current concepts and call for studies. American Journal of Respiratory and Critical Care Medicine 177(6): 567-573.

Miraglia del Giudice, M, F. Decimo, *et al.* (2006). Immune dysregulation in atopic dermatitis. Allergy and Asthma Proceedings 27(6): 451-455.

Miyawaki, T, H. Seki, *et al.* (1985). Dissociated production of IL-2 and immune (gamma) interferon by phytohaemagglutinin stimulated lymphocytes in healthy infants. Journal of Clinical Experimental Immunology 59(2): 505-511.

Molet, S, Q. Hamid, *et al.* (2001). IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. Journal of Allergy and Clinical Immunology 108(3): 430-438.

Moore K W, O'Garra A , *et al.* (1993). IL-10 Annual Review of Immunology 11: 165-190.

Moore, K. W, R. de Waal Malefyt, *et al.* (2001). IL-10 and the IL-10 receptor. Annual Reviews Immunology19: 683-765.

Mullooly, J. P, R. Schuler, *et al.* (2007). Vaccines, antibiotics, and atopy. Pharmacoepidemiology and Drug Safety 16(3): 275-288.

Munn, D. H, M. Zhou, *et al.* (1998). Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 281(5380): 1191-1193.

Nagler-Anderson, C. (2001). Man the barrier! Strategic defences in the intestinal mucosa. Nature Reviews Immunology 1(1): 59-67.

Nakae, S, S. Saijo, *et al.* (2003). IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. Proceedings of the National Academy of Sciences of the United States of America 100(10): 5986-5990.

Neaville, W. A, C. Tisler, *et al.* (2003). Developmental cytokine response profiles and the clinical and immunologic expression of atopy during the first year of life. Journal of Allergy and Clinical Immunology 112(4): 740-746.

Newburg, D. (2009). Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. Journal of Animal Science. 87(13 supplement): 26-34.

Ng, N, D. Lam, *et al.* (2006). House dust extracts have both T(H)2 adjuvant and tolerogenic activities. Journal of Allergy and Clinical Immunology 117(5): 1074-1081.

Nicaise, P, A. Gleizes, *et al.* (1998). Influence of intestinal microflora on murine bone marrow and spleen macrophage precursors. Scandinavian Journal of Immunology 48(6): 585-591.

Niers, L, R. Martin, *et al.* (2009). The effects of selected probiotic strains on the development of eczema (the PandA study). Allergy 64(9): 1349-1358.

Niers, L. E. M, H. M. Timmerman, *et al.* (2005). Identification of strong IL-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. Clinical and Experimental Allergy 35(11): 1481-1489.

Nilsson, C, A. K. Larsson, *et al.* (2004). Low numbers of IL-12-producing cord blood mononuclear cells and immunoglobulin E sensitization in early childhood. Clinical and Experimental Allergy 34(3): 373-380.

Nouri-Aria, K. T, A. M. A. Irani, *et al.* (2001). Basophil recruitment and IL-4 production during human allergen-induced late asthma. Journal of Allergy and Clinical Immunology 108(2): 205-211.

Noverr, M. C. and G. B. Huffnagel (2004). Does the microbiota regulate immune responses outside the gut? Trends in Microbiology 12(12): 562-568.

Nurse, B, M. Haus, *et al.* (1997). Reduced interferon-gamma but normal IL-4 and IL-5 release by peripheral blood mononuclear cells from Xhosa children with atopic asthma. Journal of Allergy and Clinical Immunology 100(5): 662-668.

O'Farrelly, C. (1998). Just how inflamed is the normal gut? Gut 42(5): 603-604.

Ober, C, A. Tsalenko, *et al.* (2000). A second-generation genomewide screen for asthma-susceptibility alleles in a founder population. The American Journal of Human Genetics 67(5): 1154-1162.

Oettgen, H. C. and R. S. Geha (1999). IgE in asthma and atopy: cellular and molecular connections. Journal of Clinical Investigation 104(7): 829-835.

Ogawa, T, Y. Asai, *et al.* (2006). Natural killer cell activities of synbiotic *Lactobacillus casei* ssp. casei in conjunction with dextran. Journal of Clinical Experimental Immunology 143(1): 103-109.

Ohshima, Y. and G. Delespesse (1997). T cell-derived IL-4 and dendritic cell-derived IL-12 regulate the lymphokine-producing phenotype of alloantigen-primed naive human CD4 T cells. Journal of Immunology 158(2): 629-636.

Okada H, Kuhn C, Feillet H, et al. (2010) The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. Journal of Clinical Experimental Immunology 160:1–9.

Olivares, M, M. P. Diaz-Ropero, *et al.* (2006). Antimicrobial potential of four Lactobacillus strains isolated from breast milk. Journal of Applied Microbiology 101(1): 72-79.

Ong, P. Y. and D. Y. Leung (2006). Immune dysregulation in atopic dermatitis. Current Allergy and Asthma Reports 6(5): 384-389.

Onishi, R. M. and S. L. Gaffen (2010). IL-17 and its target genes: mechanisms of IL-17 function in disease. Immunology 129(3): 311-321.

Osborn, D. A. and J. K. Sinn (2007). Probiotics in infants for prevention of allergic disease and food hypersensitivity. Cochrane Database of Systematic Reviews(4).

Ostman, S, C. Rask, *et al.* (2006). Impaired regulatory T cell function in germ-free mice. European Journal of Immunology 36(9): 2336-2346.

Ouwehand, A, E. Isolauri, *et al.* (2002). The role of the intestinal microflora for the development of the immune system in early childhood. European Journal of Nutrition 41 Supplement 1: I32-137.

Oyoshi, M. K, R. He, *et al.* (2009). Cellular and molecular mechanisms in atopic dermatitis. Advances in Immunology 102: 135-226.

Pabst, R. (1991). Lymphocyte Migration to the Gut - Oversimplifications and Controversial Aspects. Immunologic Research 10(3-4): 279-281.

Paganelli, R, S. Ciuffreda, *et al.* (2002). Probiotics and food-allergic diseases. Allergy 57 Suppl 72: 97-99.

Palmer, L. J, S. E. Daniels, *et al.* (1998). Linkage of chromosome 5q and 11q gene markers to asthma-associated quantitative traits in Australian children. American Journal of Respiratory and Critical Care Medicine 158(6): 1825-1830.

Pan, S. J, C. H. Kuo, *et al.* (2010). Probiotics and allergy in children - An update review. Pediatric Allergy and Immunology 21(4): E659-E666.

Park, H, Z. Li, *et al.* (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing IL 17. Nature Immunology 6(11): 1133-1141.

Parronchi, P, F. Brugnolo, *et al.* (1999). Phosphorothioate oligodeoxynucleotides promote the *in vitro* development of human allergen-specific $CD4^+$ T cells into Th1 effectors. Journal of Immunology 163(11): 5946-5953.

Pascual, V, Y. J. Liu, *et al.* (1994). Analysis of somatic mutation in five B cell subsets of human tonsil. Journal of Experimental Medicine 180(1): 329-339.

Paterson, W. G. and K. A. Grant (1975). Advanced intraligamentous pregnancy. Report of a case, review of the literature and a discussion of the biological implications. Obstetrical and Gynecological Survey 30(11): 715-726.

Patni, S, P. Flynn, *et al.* (2007). An introduction to Toll-like receptors and their possible role in the initiation of labour. Bjog-an International Journal of Obstetrics and Gynaecology 114(11): 1326-1334.

Paul, W. E. and R. A. Seder (1994). Lymphocyte-Responses and Cytokines. Cell 76(2): 241-251.

Paul, W. E. and J. F. Zhu (2010). How are T(H)2-type immune responses initiated and amplified? Nature Reviews Immunology 10(4): 225-235.

Peisong, G. (2001). Epidemiology of Asthma and Atopy: Environmental and Genetic Factors.

Pelucchi C. Chatenoud *et al.* (2012). Probiotics supplementation during pregnancy or infancy for the prevention of atopic dermatitis: a meta-analysis. Epidemiology (23):402-414.

Penders, J, C. Thijs, *et al.* (2006). Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118(2): 511-521.

Peran, L, D. Camuesco, *et al.* (2005). Preventative effects of a probiotic, *Lactobacillus salivarius* ssp. salivarius, in the TNBS model of rat colitis. World Journal of Gastroenterology 11(33): 5185-5192.

Perez, O, D. Mitchell, *et al.* (2005). Multiparameter analysis of intracellular phosphoepitopes in immunophenotyped cell populations by flow cytometry. Current Protocols in Cytometry (6): 20.

Peroni, D. G, L. Pescollderungg, *et al* (2010). Immune regulatory cytokines in the milk of lactating women from farming and urban environments. Journal of Pediatric Allergy and Immunology 21(6): 977-982.

Pessi T, Sue Tas Y, *et al.* (2000). IL-10 generation in atopic children following oral *Lactobacillus rhamnosus* GG Clinical and Experimental Allergy 30: 1804-1808.

Pfefferle, P. I, O. Pinkenburg, *et al.* (2010). Fetal Epigenetic Mechanisms and Innate Immunity in Asthma. Current Allergy and Asthma Reports 10(6): 434-443.

Phuapradit, P, W. Varavithya, *et al.* (1999). Reduction of rotavirus infection in children receiving bifidobacteria-supplemented formula. Journal of the Medical Association of Thailand 82 Supplement 1: S43-48.

Piccinni, M. P, C. Scaletti, *et al.* (2000). Role of hormone-controlled Th1-and Th2-type cytokines in successful pregnancy. Journal of Neuroimmunology 109(1): 30-33.

Picker, L. J, L. W. Terstappen, *et al.* (1990). Differential expression of homing-associated adhesion molecules by T cell subsets in man. Journal of Immunology 145(10): 3247-55.

Pierre, P (2002). Latic acid bacteria inhibit Th2 cytokine production by mononuclear cells from allergic patients. Jounral of Allergy and Clinical Immunology 110(4): 617-623.

Pirenne-Ansart, H, F. Paillard, *et al.* (1995). Defective cytokine expression but adult-type T-cell receptor, CD8, and p56lck modulation in CD3- or CD2-activated T cells from neonates. Pediatric Research 37(1): 64-69.

Plebanski, M, M. Saunders, *et al.* (1992). Primary and Secondary Human Invitro T-Cell Responses to Soluble-Antigens Are Mediated by Subsets Bearing Different CD45 Isoforms. Immunology 75(1): 86-91.

Pochard, P, P. Gosset, *et al.* (2002). Lactic acid bacteria inhibit TH2 cytokine production by mononuclear cells from allergic patients. Journal of Allergy and Clinical Immunology 110(4): 617-623.

Pohjavuori, E, M. Viljanen, *et al.* (2004). *Lactobacillus* GG effect in increasing IFN-gamma production in infants with cow's milk allergy. Journal of Allergy and Clinical Immunology 114(1): 131-136.

Possin, M. E, S. Morgan, *et al.* (2010). The relationships among immunoglobulin levels, allergic sensitization, and viral respiratory illnesses in early childhood. Pediatric Allergy and Immunology 21(6): 990-996.

Poupard, J. A, I. Husain, *et al.* (1973). Biology of the bifidobacteria. Bacteriology Reviews 37(2): 136-165.

Power, L. L, E. J. Popplewell, *et al.* (2002). Immunoregulatory molecules during pregnancy and at birth. Journal of Reproductive Immunology 56(1-2): 19-28.

Pranke, P, R. R. Failace, *et al.* (2001). Hematologic and immunophenotypic characterization of human umbilical cord blood. Acta Haematologica 105(2): 71-76.

Prause, O, S. Bozinovski, *et al.* (2004). Increased matrix metalloproteinase-9 concentration and activity after stimulation with IL-17 in mouse airways. Thorax 59(4): 313-317.

Prefontaine, D, S. Lajoie-Kadoch, *et al.* (2009). Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. Journal of Immunology 183(8): 5094-5103.

Prescott, S, D. Martino, *et al.* (2010). Progress in understanding postnatal Immune dysregulation in allergic disease. World Allergy Organization 3 (4): 162-166.

Prescott, S. and A. Nowak-Wegrzyn (2011) Strategies to prevent or reduce allergic disease. Annals of Nutrition and Metabolism 59 Suppl 1: 28-42.

Prescott S. L, Tavlor A, *et al.* (2003). Neonatal IL-12 capacity is associated with variations in allergen-specific immune responses in the neonatal and postnatal periods Clinical and Experimental Allergy 33: 566-572.

Prescott, S. L. (2003). Early origins of allergic disease: a review of processes and influences during early immune development. Current Opinion in Allergy and Clinical Immunology 3(2): 125-132.

Prescott, S. L. and B. Bjorksten (2007). Probiotics for the prevention or treatment of allergic diseases. Journal of Allergy and Clinical Immunology 120(2): 255-262.

Prescott, S. L. and J. A. Dunstan (2005). Immune dysregulation in allergic respiratory disease: the role of T regulatory cells. Pulmonary Pharmacology & Therapeutics 18(3): 217-228.

Prescott, S. L. Dunstan, *et al.* (2005). Clinical effects of probiotics are associated with increased interferon-gamma responses in very young children with atopic dermatitis. Clinical and Experimental Allergy 35(12): 1557-1564.

Prescott, S. L, C. Macaubas, *et al.* (1998). Transplacental priming of the human immune system to environmental allergens: Universal skewing of initial T cell responses toward the Th2 cytokine profile. Journal of Immunology 160(10): 4730-4737.

Prescott, S. L, C. Macaubas, *et al.* (1999). Development of allergen-specific T-cell memory in atopic and normal children. Lancet 353(9148): 196-200.

Prescott, S. L, A. Taylor, *et al.* (2003). Neonatal IL-12 capacity is associated with variations in allergen-specific immune responses in the neonatal and postnatal periods. Clinical and Experimental Allergy 33(5): 566-572.

Prescott, S. L, K. Wickens, *et al.* (2008). Supplementation with *Lactobacillus rhamnosus* or Bifidobacterium lactis probiotics in pregnancy increases cord blood interferon-gamma and breast milk transforming growth factor-beta and immunoglobin A detection. Clinical and Experimental Allergy 38(10): 1606-1614.

Pritchard, M. A, E. Baker, *et al.* (1991). The IL-4 receptor gene (IL4R) maps to 16p11.2-16p12.1 in human and to the distal region of mouse chromosome 7. Genomics 10(3): 801-806.

Provoost, S, T. Maes, *et al.* (2009). Decreased FOXP3 protein expression in patients with asthma. Allergy 64(10): 1539-1546.

Pujol, J. L, B. Cosso, *et al.* (1990). IL-1 Release by Alveolar Macrophages in Asthmatic-Patients and Healthy-Subjects. International Archives of Allergy and Applied Immunology 91(2): 207-210.

Purwar, R, J. Campbell, *et al.* (2011). Resident Memory T Cells (T-RM) Are Abundant in Human Lung: Diversity, Function, and Antigen Specificity. PLoS One 6(1).

Raedler, D, S. Illi, *et al.* (2012). IL-10 polymorphisms influence neonatal immune responses, atopic dermatitis, and wheeze at age 3 years. Journal of Allergy and Clinical Immunology

Raes, M, P. Alliet, *et al.* (1993). Lymphocyte Subpopulations in Healthy Newborn-Infants - Comparison of Cord-Blood Values with Values 5 Days after Birth. Journal of Pediatrics 123(3): 465-467.

Redman, C. W. G. and I. L. Sargent (2008). Circulating microparticles in normal pregnancy and pre-eclampsia. Placenta 29: S73-S77.

Reece, P, A. Thanendran, *et al.* (2011). Maternal allergy modulates cord blood hematopoietic progenitor Toll-like receptor expression and function. Journal of Allergy and Clinical Immunology 127(2): 447-453.

Renz, H, P. Brandtzaeg, *et al.* (2011). The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. Nature Reviews Immunology 12(1): 9-23.

Rettger, L. F, W. N. Levy, *et al.* (1935). *Lactobacillus acidophilus* and its therapeutic application. American Journal of Public Health Nations Health 25(9): 1056–1057.

Rinne, M, M. Kalliomaki, *et al.* (2005). Effect of probiotics and breastfeeding on the bifidobacterium and lactobacillus/enterococcus microbiota and humoral immune responses. Journal of Pediatrics 147(2): 186-191.

Romagnani, S. (2000). The role of lymphocytes in allergic disease. Journal of Allergy and Clinical Immunology 105(3): 399-408.

Romagnani, S. (2004). The increasing prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? Immunology 112(3): 352-363.

Rosalind, S. and S. Bloomfield (2004). The hygiene hypothesis and implications for home hygiene. The International Scientific Forum on Home Hygiene: 1-218.

Rosenfeldt, V, E. Benfeldt, *et al.* (2003). Effect of probiotic Lactobacillus strains in children with atopic dermatitis. Journal of Allergy and Clinical Immunology 111(2): 389-395.

Roth, I, D. B. Corry, *et al.* (1996). Human placental cytotrophoblasts produce the immunosuppressive cytokine IL 10. Journal of Experimental Medicine 184(2): 539-548.

Sacks, G, I. Sargent, *et al.* (1999). An innate view of human pregnancy. Immunology Today 20(3): 114-118.

Sakaguchi, S. (2004). Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annual Review of Immunology 22: 531-562.

Sanders, M. E, M. W. Makgoba, *et al.* (1988). Human Naive and Memory T-Cells - Reinterpretation of Helper-Inducer and Suppressor-Inducer Subsets. Immunology Today 9(7-8): 195-199.

Sandford, A. J, T. Chagani, *et al.* (2000). Polymorphisms in the IL-4, IL-4RA, and FCERIB genes and asthma severity. Journal of Allergy and Clinical Immunology 106(1 Pt 1): 135-140.

Sargent, I. L, A. M. Borzychowski, *et al.* (2006). NK cells and human pregnancy - an inflammatory view. Trends in Immunology 27(9): 399-404.

Sashihara, T, N. Sueki, *et al.* (2006). An analysis of the effectiveness of heat-killed lactic acid bacteria in alleviating allergic diseases. Journal of Dairy Science 89(8): 2846-2855.

Satokari, R, T. Gronroos, *et al.* (2009). Bifidobacterium and Lactobacillus DNA in the human placenta. Letters in Applied Microbiobiology 48(1): 8-12.

Schaerli, P, L. Ebert, *et al.* (2004). A skin-selective homing mechanism for human immune surveillance T cells. Journal of Experimental Medicine 199(9): 1265-1275.

Schaub, B, J. Liu, *et al.* (2008). Impairment of T-regulatory cells in cord blood of atopic mothers. Journal of Allergy and Clinical Immunology 121(6): 1491-1499.

Schmidt-Weber, C. B, M. Akdis, *et al.* (2007). TH17 cells in the big picture of immunology. Journal of Allergy and Clinical Immunology 120(2): 247-254.

Schmidt-Weber, C. B. and K. Blaser (2004). Regulation and role of transforming growth factor-beta in immune tolerance induction and inflammation. Current Opinion in Immunology 16(6): 709-716.

Schnyder-Candrian, S, D. Togbe, *et al.* (2006). IL-17 is a negative regulator of established allergic asthma. Journal of Experimental Medicine 203(12): 2715-2725.

Schrezenmeir, J. and M. de Vrese (2001). Probiotics, prebiotics, and synbiotics--approaching a definition. American Journal of Clinical Nutrition 73(2 Suppl): 361S-364S.

Schwonzen , M, V. Diehl, *et al.* (2007). Immunophenotyping of surface antigens in acute myeloid leukemia by flow cytometry after red blood cell lysis. Leukemia Research. 31(1): 113-116.

Seddiki, N, B. Santner-Nanan, *et al.* (2006). Expression of IL-2 and IL-7 receptors discriminates between human regulatory and activated T cells. Journal of Experimental Medicine 203(7): 1693-1700.

Seder, R. A. and W. E. Paul (1994). Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. Annual Reviews Immunology 12: 635-673.

Sedman, P. C, J. Macfie, *et al.* (1994). The Prevalence of Gut Translocation in Humans. Gastroenterology 107(3): 643-649.

Sekirov, I, S. L. Russell, *et al.* (2010). Gut Microbiota in Health and Disease. Physiological Reviews 90(3): 859-904.

Sepp, E, K. Julge, *et al.* (1997). Intestinal microflora of Estonian and Swedish infants. Acta Paediatrica 86(9): 956-961.

Sergejeva, S, S. Ivanov, *et al.* (2005). IL-17 as a recruitment and survival factor for airway macrophages in allergic airway inflammation. American Journal of Respiratory Cell and Molecular Biology 33(3): 248-253.

Sheridan, B. S. and L. Lefrancois (2011). Regional and mucosal memory T cells. Nature Immunology 12(6): 485-491.

Shida, K. (2006). Essential Roles of Monocytes in Stimulating Human Peripheral BloodMononuclear Cells with *Lactobacillus casei* To Produce Cytokines and Augment Natural Killer Cell Activity. Immunology 13: 997-1003.

Shirakawa, I, K. A. Deichmann, *et al.* (2000). Atopy and asthma: genetic variants of IL-4 and IL-13 signalling. Immunology Today 21(2): 60-64.

Shore, S. A. and P. E. Moore (2002). Effects of cytokines on contractile and dilator responses of airway smooth muscle. Clinical and Experimental Pharmacology and Physiology 29(10): 859-866.

Shreiner, A, G. B. Huffnagle, *et al.* (2008). The Microflora Hypothesis of allergic disease. Advances in Experimental Medicine and Biology 635: 113-134.

Shroff, K. E, K. Meslin, *et al.* (1995). Commensal Enteric Bacteria Engender a Self-Limiting Humoral Mucosal Immune-Response While Permanently Colonizing the Gut. Infection and Immunity 63(10): 3904-3913.

Siegrist, C. A. (2001). Neonatal and early life vaccinology. Vaccine 19(25-26): 3331-3346.

Sierra, S, F. Lara-Villoslada, *et al.* (2010). Intestinal and immunological effects of daily oral administration of *Lactobacillus salivarius* CECT5713 to healthy adults. Anaerobe 16(3): 195-200.

Sigmundsdottir, H. and E. C. Butcher (2008). Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. Nature Immunology (9): 981-987.

Silen, M. L, A. Firpo, *et al.* (1989). IL-1α and TNF-α cause placental injury in the rat. American Journal of Pathology 135(2): 239-244.

Silver, E, H. Yin-DeClue, *et al.* (2009). Lower levels of plasmacytoid dendritic cells in peripheral blood are associated with a diagnosis of asthma 6 yr after severe respiratory syncytial virus bronchiolitis. Pediatric Allergy and Immunology 20(5): 471-476.

Silver, R. M, W. S. Lohner, *et al.* (1994). Lipopolysaccharide-induced fetal death: the role of tumor-necrosis factor alpha. Biology of Reproduction 50(5): 1108-1112.

Sims, G. P, R. Ettinger, *et al.* (2005). Identification and characterization of circulating human transitional B cells. Blood 105(11): 4390-4398.

Sjogren, Y. M, S. Tomicic, *et al.* (2009). Influence of early gut microbiota on the maturation of childhood mucosal and systemic immune responses. Clinical and Experimental Allergy 39(12): 1842-1851.

Skov, L. and O. Baadsgaard (2000). Bacterial superantigens and inflammatory skin diseases. Clinical and Experimental Dermatology 25(1): 57-61.

Skov, L, J. V. Olsen, *et al.* (2000). Application of staphylococcal enterotoxin B on normal and atopic skin induces up-regulation of T cells by a superantigen-mediated mechanism. Journal of Allergy and Clinical Immunology 105(4): 820-826.

Smart, J. M. and A. S. Kemp (2001). Ontogeny of T-helper 1 and T-helper 2 cytokine production in childhood. Pediatric Allergy and Immunology 12(4): 181-187.

Smart, J. M. and A. S. Kemp (2002). Increased Th1 and Th2 allergen-induced cytokine responses in children with atopic disease. Clinical and Experimental Allergy 32(5): 796-802.

Smith, M, M. R. Tourigny, *et al.* (2008). Children with egg allergy have evidence of reduced neonatal $CD4^{+}CD25^{+}CD127^{lo/-}$ regulatory T cell function. Journal of Allergy and Clinical Immunology 121(6): 1460-1466.

Smits, H. H, B. Everts, *et al.* (2010). Chronic helminth infections protect against allergic diseases by active regulatory processes. Current Allergy and Asthma Reports 10(1): 3-12.

Soh, S. E, M. Aw, *et al.* (2009). Probiotic supplementation in the first 6 months of life in at risk Asian infants - effects on eczema and atopic sensitization at the age of 1 year. Clinical and Experimental Allergy 39(4): 571-578.

Sohn, M. H, J. S. Song, *et al.* (2007). Association of IL-10 gene promoter polymorphism in children with atopic dermatitis. Journal of Pediatrics 150(1): 106-108.

Sokol, C. L, G. M. Barton, *et al.* (2008). A mechanism for the initiation of allergen-induced T helper type 2 responses. Nature Immunology 9(3): 310-318.

Solway, J. and C. G. Irvin (2007). Airway smooth muscle as a target for asthma therapy. New England Journal of Medicine 356(13): 1367-1369.

Spergel, J. M. and A. S. Paller (2003). Atopic dermatitis and the atopic march. Journal of Allergy and Clinical Immunology 112(6 Suppl): S118-27.

Sporik, R, S. T. Holgate, *et al.* (1990). Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood. A prospective study.New England Journal of Medicine 323(8): 502-507.

Starling, J. R. and E. Balish (1981). Lysosomal Enzyme-Activity in Pulmonary Alveolar Macrophages from Conventional, Germ-Free, Monoassociated, and Conventionalized Rats. Journal of the Reticuloendothelial Society 30(6): 497-505.

Strachan DP. (1989). Hay fever, hygiene, and household size. British Medical Journal 299(6710): 1259-60.

Strachan, D. P. (2000). Family size, infection and atopy: the first decade of the hygiene hypothesis. Thorax 55 Suppl 1: S2-S10.

Sudo, N, S. Sawamura, *et al.* (1997). The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. Journal of Immunology 159(4): 1739-1745.

Supramac, L. (2008). How the immune system works.

Szabo SJ, Sullivan BM, , Peng SL (2003). Molecular mechanisms regulating Th1 immune responses. Annual Reviews Immunology(21): 713-758.

SzekeresBartho, J, Z. Faust, *et al.* (1996). The immunological pregnancy protective effect of progesterone is manifested via controlling cytokine production. American Journal of Reproductive Immunology 35(4): 348-351.

Tallon, D. F, D. J. Corcoran, *et al.* (1984). Circulating lymphocyte subpopulations in pregnancy: a longitudinal study. Journal of Immunology 132(4): 1784-1797.

Tang, M. and A. Kemp (1994). Production and secretion of interferon-gamma in children with atopic dermatitis. Journal of Clinical Experimental Immunology 95(1): 66-72.

Tang, M, A. Kemp, *et al.* (1993). IL-4 and interferon-gamma production in children with atopic disease. Journal of Clinical Experimental Immunology 92(1): 120-124.

Tang, M. L. (2009). Probiotics and prebiotics: immunological and clinical effects in allergic disease. Nestle Nutrition Workshop Series Pediatriac Program 64: 219-235; discussion 235-238, 251-257.

Tang, M. L, J. Coleman, *et al.* (1995). IL-4 and interferon-gamma production in atopic and non-atopic children with asthma. Clinical and Experimental Allergy 25(6): 515-521.

Tang, M. L. K. and A. S. Kemp (1995). Ontogeny of IL-4 Production. Pediatric Allergy and Immunology 6(1): 11-19.

Tang, M. L. K, A. S. Kemp, *et al.* (1994). Reduced Interferon-Gamma Secretion in Neonates and Subsequent Atopy. Lancet 344(8928): 983-985.

Tang, M. L. K, S. J. Lahtinen, *et al.* (2009). Prenatal Administration of *Lactobacillus rhamnosus* GG Can Modulate Intestinal Microbiota In Infants At High Risk Of Allergic Disease. Journal of Allergy and Clinical Immunology 123(2): S224-S224.

Tannock, G. W. (1999). Identification of lactobacilli and bifidobacteria. Current Issues in Molecular Biology 1(1-2): 53-64.

Taylor, A, J. Hale, *et al.* (2006). Evaluation of the effects of probiotic supplementation from the neonatal period on innate immune development in infancy. Clinical and Experimental Allergy 36(10): 1218-1226.

Taylor, A. L, J. A. Dunstan, *et al.* (2007). Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: A randomized controlled trial. Journal of Allergy and Clinical Immunology 119(1): 184-191.

Taylor, A. L, J. Hale, *et al.* (2006). Effects of probiotic supplementation for the first 6 months of life on allergen- and vaccine-specific immune responses. Clinical and Experimental Allergy 36(10): 1227-1235.

Teixeira, L. K, B. P. F. Fonseca, *et al.* (2005). The role of interferon-gamma on immune and allergic responses. Memorias Do Instituto Oswaldo Cruz 100: 137-144.

Ten Dam, G. B, C. F. Zilch, *et al.* (2000). Regulation of alternative splicing of CD45 by antagonistic effects of SR protein splicing factors. Journal of Immunology 164(10): 5287-5295.

Terness, P, M. Kallikourdis, *et al.* (2007). Tolerance signaling molecules and pregnancy: IDO, galectins, and the renaissance of regulatory T cells. American Journal of Reproductive Immunology 58(3): 238-254.

Teta, J (2006). Exercise is medicine: the anti-inflammatory effects of high intensity exercise. Townsend Letter for Doctors and Patients (November): 1-10.

Thaxton, J. E. and S. Sharma (2010). IL-10: a multi-faceted agent of pregnancy. Am Journal of Reproductive Immunology 63(6): 482-491.

Thornton, C. A, J. A. Holloway, *et al.* (2003). Fetal exposure to intact immunoglobulin E occurs via the gastrointestinal tract. Clinical and Experimental Allergy 33(3): 306-311.

Thornton, C. A, T. V. Macfarlane, *et al.* (2010). The Hygiene Hypothesis Revisited: Role of Materno-Fetal Interactions. Current Allergy and Asthma Reports 10(6): 444-452.

Thulesen, S, A. Jorgensen, *et al.* (1999). Superantigens are presented by and activate thymocytes from infants. Experimental and Clinical Immunogenetics 16(4): 226-233.

Tighe, H, K. Takabayashi, *et al.* (2000). Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. Journal of Allergy and Clinical Immunology 106(1): 124-134.

Timmerman HM, Koning CJ *et al.* (2004) Monostrain, multistrain and multispecies probiotics A comparison of functionality and efficacy. International Journal of Food Microbiology (96):219–233.

Tissier, H. (1900). Recherchers sur la flora intestinale normale et pathologique du nourisson.Thesis, University of Paris,France

Trinchieri, G. (1995). IL-12 - a Proinflammatory Cytokine with Immunoregulatory Functions That Bridge Innate Resistance and Antigen-Specific Adaptive Immunity. Annual Review of Immunology 13: 251-276.

Tulic, M. K, M. Hodder, *et al.* (2011) Differences in innate immune function between allergic and nonallergic children: new insights into immune ontogeny. Journal of Allergy and Clinical Immunology 127(2): 470-478.

Turner, H. and J. P. Kinet (1999). Signalling through the high-affinity IgE receptor Fc epsilon RI. Nature 402(6760): B24-B30.

Uchida, Y, K. Kurasawa, *et al.* (2001). Increase of dendritic cells of type 2 (DC2) by altered response to IL-4 in atopic patients. Journal of Allergy and Clinical Immunology 108(6): 1005-1011.

Uings, I, I. Puxeddu, *et al.* (2005). Effects of dexamethasone on TNF-alpha-induced release of cytokines from purified human blood eosinophils. Clinical Molecular Allergy 3(1): 5.

Umesaki, Y, H. Setoyama, *et al.* (1999). Differential roles of segmented filamentous bacteria and clostridia in development of the intestinal immune system. Infection and Immunity 67(7): 3504-3511.

Upham J, Lee P, *et al.* (2002). Development of IL-12-Producing Capacity throughout Childhood. Infection and Immunity 70(12): 6583-6588.

Upham, J. W, L. M. Hayes, *et al.* (1999). Reduced expression of hemopoietic cytokine receptors on cord blood progenitor cells in neonates at risk for atopy. Journal of Allergy and Clinical Immunology 104(2): 370-375.

Upham, J. W, P. G. Holt, *et al.* (2004). HLA-DR expression on neonatal monocytes is associated with allergen-specific immune responses. Journal of Allergy and Clinical Immunology 114(5): 1202-1208.

Upham, J. W, P. T. Lee, *et al.* (2002). Development of IL-12-producing capacity throughout childhood. Infection and Immunity 70(12): 6583-6588.

Upham, J. W, G. C. Zhang, *et al.* (2009). Plasmacytoid dendritic cells during infancy are inversely associated with childhood respiratory tract infections and wheezing. Journal of Allergy and Clinical Immunology 124(4): 707-713.

Urry, Z, E. Xystrakis, *et al.* (2006). IL-10-secreting regulatory T cells in allergy and asthma. Current Allergy and Asthma Reports 6(5): 363-371.

Vaarala, O. (2003). Immunological effects of probiotics with special reference to lactobacilli. Clinical and Experimental Allergy 33(12): 1634-1640.

van der Velden, V. H, M. P. Laan, *et al.* (2001). Selective development of a strong Th2 cytokine profile in high-risk children who develop atopy: risk factors and regulatory role of IFN-gamma, IL-4 and IL-10. Clinical and Experimental Allergy 31(7): 997-1006.

van Gent, R, C. M. van Tilburg, *et al.* (2009). Refined characterization and reference values of the pediatric T- and B-cell compartments. Clinical Immunology 133(1): 95-107.

van Rijt, L. S, S. Jung, *et al.* (2005). *In vivo* depletion of lung $CD11c^+$ dendritic cells during allergen challenge abrogates the characteristic features of asthma. Journal of Experimental Medicine 201(6): 981-991.

Vercelli, D. (2006). Mechanisms of the hygiene hypothesis--molecular and otherwise. Current Opinion in Immunology 18(6): 733-737.

Vezys, V, A. Yates, *et al.* (2009). Memory CD8 T-cell compartment grows in size with immunological experience. Nature 457(7226): 196-199.

Vigano, A, S. Esposito, *et al.* (1999). Differential development of type 1 and type 2 cytokines and beta-chemokines in the ontogeny of healthy newborns. Biology of the Neonate 75(1): 1-8.

Viljanen, M, E. Pohjavuori, *et al.* (2005). Induction of inflammation as a possible mechanism of probiotic effect in atopic eczema-dermatitis syndrome. Journal of Allergy and Clinical Immunology 115(6): 1254-1259.

Vitoratos, N, C. Papadias, *et al.* (2006). Elevated circulating IL-1beta and TNF-alpha, and unaltered IL-6 in first-trimester pregnancies complicated by threatened abortion with an adverse outcome. Mediators of Inflammation 2006(4): 1-6.

von Bubnoff, D, D. Hanau, *et al.* (2003). Indoleamine 2,3-dioxygenase-expressing antigen-presenting cells and peripheral T-cell tolerance: Another piece to the atopic puzzle? Journal of Allergy and Clinical Immunology 112(5): 854-860.

von der Weid T, Bulliard C, *et al.* (2001). Induction by a lactic acid bacterium of a population of $CD4^+$ T cells with low proliferative capacity that produce transforming growth factor beta and IL-10 Clinical and Diagnostic Laboratory Immunology 4: 695-701.

von der Weid, T, C. Bulliard, *et al.* (2001). Induction by a lactic acid bacterium of a population of $CD4^+$ T cells with low proliferative capacity that produce transforming growth factor beta and IL-10. Clinical and Diagnostic Laboratory Immunology 8(4): 695-701.

von Hertzen, L. and T. Haahtela (2006^a). Disconnection of man and the soil: reason for the asthma and atopy epidemic? Journal of Allergy and Clinical Immunology 117(2): 334-344.

von Hertzen, L. and T. Haahtela (2004^b). Asthma and atopy - the price of affluence? Allergy 59(2): 124-137.

Wadonda-Kabondo, N, J. A. Sterne, *et al.* (2003). A prospective study of the prevalence and incidence of atopic dermatitis in children aged 0-42 months. British Journal of Dermatology 149(5): 1023-1028.

Wahl, S. M. (1992). Transforming Growth-Factor-Beta in Inflammation - a Cause and a Cure. Journal of Clinical Immunology 12(2): 61-74.

Wakim, L. M, J. Waithman, *et al.* (2008). Dendritic cell-induced memory T cell activation in nonlymphoid tissues. Science 319(5860): 198-202.

Wakkach, A, N. Fournier, *et al.* (2003). Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation *in vivo*. Immunity 18(5): 605-617.

Walton, K. L. W, J. P. He, *et al.* (2006). Dendritic cells in germ-free and specific pathogen-free mice have similar phenotypes and *in vitro* antigen presenting function. Immunology Letters 102(1): 16-24.

Warner, J. A, E. A. Miles, *et al.* (1994). Is deficiency of interferon gamma production by allergen triggered cord blood cells a predictor of atopic eczema? Clinical and Experimental Allergy 24(5): 423-430.

Warner, J. O, J. A. Warner, *et al.* (1994). Reduced Interferon-Gamma Secretion in Neonates and Subsequent Atopy. Lancet 344(8935): 1516-1516.

Watanabe, M, Y. Iwatani, *et al.* (1997). Changes in T, B, and NK lymphocyte subsets during and after normal pregnancy. American Journal of Reproductive Immunology 37(5): 368-377.

Watanabe, S, Y. Narisawa, *et al.* (2003). Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. Journal of Allergy and Clinical Immunology 111(3): 587-591.

Wegmann, T. G, H. Lin, *et al.* (1993). Bidirectional Cytokine Interactions in the Maternal-Fetal Relationship - Is Successful Pregnancy a Th2 Phenomenon. Immunology Today 14(7): 353-356.

West, C. E, M. L. Hammarstrom, *et al.* (2009). Probiotics during weaning reduce the incidence of eczema. Pediatric Allergy and Immunology 20(5): 430-437.

Whelan, R, C. Kim, *et al.* (2004). Role and regulation of IL-1 molecules in pro-asthmatic sensitised airway smooth muscle. European Respiratory Journal 24(4): 559-567.

White, G. P, P. M. Watt, *et al.* (2002). Differential patterns of methylation of the IFN-gamma promoter at CpG and Non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult $CD45RO^-$ T cells. Journal of Immunology 168(6): 2820-2827.

Whitley, G. S. J, P. R. Dash, *et al.* (2007). Increased apoptosis in first trimester extravillous trophoblasts from pregnancies at higher risk of developing preeclampsia. American Journal of Pathology 170(6): 1903-1909.

Whorwell, P. J, L. Altringer, *et al.* (2006). Efficacy of an encapsulated probiotic *Bifidobacterium infantis* 35624 in women with irritable bowel syndrome. American Journal of Gastroenterology 101(7): 1581-1590.

Wickens, K, P. N. Black, *et al.* (2008). A differential effect of 2 probiotics in the prevention of eczema and atopy: a double-blind, randomized, placebo-controlled trial. Journal of Allergy and Clinical Immunology 122(4): 788-794.

Wickens, K, N. Pearce, *et al.* (1999). Antibiotic use in early childhood and the development of asthma. Clinical and Experimental Allergy 29(6): 766-771.

Williams, M. B. and E. C. Butcher (1997). Homing of naive and memory T lymphocyte subsets to Peyer's patches, lymph nodes, and spleen. Journal of Immunology 159(4): 1746-1752.

Williams, P. J, R. F. Searle, *et al.* (2009). Decidual leucocyte populations in early to late gestation normal human pregnancy. Journal of Reproductive Immunology 82(1): 24-31.

Williams, T. J, C. A. Jones, *et al.* (2000). Fetal and neonatal IL-13 production during pregnancy and at birth and subsequent development of atopic symptoms. Journal of Allergy and Clinical Immunology 105(5): 951-959.

Wilson, C. B. (1991). The Ontogeny of Lymphocyte-T Maturation and Function. Journal of Pediatrics 118(3): S4-S9.

Wilson, C. B, J. Westall, *et al.* (1986). Decreased Production of Interferon-Gamma by Human Neonatal Cells - Intrinsic and Regulatory Deficiencies. Journal of Clinical Investigation 77(3): 860-867.

Wjst, M, S. Kruse, *et al.* (2002). Asthma and IL-4 receptor alpha gene variants. European Journal of Immunogenetics 29(3): 263-268.

Wynn (2003) IL-13 Effector functions Annual Review of Immunology volume 21: 425-456

Wüthrich B, Schmid-Grendelmeier P *et al.* (2003) The atopic eczema/dermatitis syndrome. Epidemiology, natural course, and immunology of the IgE-associated ("extrinsic") and the nonallergic ("intrinsic") AEDS. Journal of Investigational Allergology and Clinical Immunology; 13:1–5.

Yalkult (2010). Yakult company profile 2009 - 2010. **http://www.yakult.co.jp/english/pdf/profile2009-2010.pdf.**

Yang, M, S. P. Hogan, *et al.* (2003). Eotaxin-2 and IL-5 cooperate in the lung to regulate IL-13 production and airway eosinophilia and hyperreactivity. Journal of Allergy and Clinical Immunology 112(5): 935-943.

Yang, X, J. J. Letterio, *et al.* (1999). Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. EMBO Journal 18(5): 1280-1291.

Yasui, H, A. Mike, *et al.* (1989). Immunogenicity of Bifidobacterium breve and change in antibody production in Peyer's patches after oral administration. Journal of Dairy Science 72(1): 30-35.

Yoo J, Tcheurekdjian H, Lynch SV, *et al.* (2007) Microbial manipulation of immune function for asthma prevention: inferences from clinical trials. Proceedings of the American Thoracic Society 4:277–282.

Yui J, Garcia-Lloret MI, *et al.* (1993). Disruption of trophoblast monolayers by TNF-alpha and IFN-gamma and antagonism by GM-CSF. Serono Symposia: 174-179.

Zeuthen, L. H, L. N. Fink, *et al.* (2008). Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. Immunology 123(2): 197-208.

Zhao, J, Z. Lei, *et al.* (2009). Human Pregnancy Up-Regulates Tim-3 in Innate Immune Cells for Systemic Immunity. Journal of Immunology 182(10): 6618-6624.